SYMPOSIUM ON THE BIOLOGY OF BACTERIAL SPORES[1]

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INTRODUCTION

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Bacterial spores have been of interest to microbiologists since the beginning of observations of bacteria. They have afforded grounds for speculation and

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[3] Abstract prepared by the author.

[4] Because of limitations of space it has been necessary to restrict references. An unabridged list of references pertinent to this section of the review may be obtained from the author.

experimentation as to: their nature and biology; the mode of, and factors influencing, their formation; their germination history; and especially, their resistance to adverse environments. They have been intriguing both to investigators whose interests were in "pure" bacteriology, and to those with interests chiefly in some applied phase of the subject. Certain spore bearing species of bacteria are of much importance in pathogenic bacteriology, both as it relates to humans and to animals. Others are of main concern in industrial processes in which microorganisms are, or may be, involved. One can conclude that there is no area in the entire field of bacteriology in which spores are not of importance.

The amount and type of research in recent years on each of the various topics of spore investigation indicate a growing recognition and appreciation of the necessity of basic information in this field. The subjects which were selected for presentation at the symposium were chosen after much consideration with the view of providing a well balanced program with something of value from both theoretical and practical viewpoints. Each participant has made significant contributions in the field of his specialty.

Part I. Biological Role of Spores

CARL LAMANNA

A discussion of the biological role of spores might well begin with a brief outline of their chemical structure. For it is among these properties one hopes to find clues as to the possible roles in nature for which spores are adapted. Unfortunately, bacterial endospores in their chemical composition have revealed no unusual properties. Their total water and ash content is like that of vegetative cells, except for a higher percentage of calcium. The dry weight nitrogen content is somewhat higher than in vegetative cells. They possess a full complement of the naturally occurring amino acids. There are no obvious clues in chemical composition to suggest the basis for the endospore's unique physiological properties (20, 21, 30, 44, 58, 95).

Studies of antigenic structure prove that endospores like all other living things are composed of a mosaic of antigens. While some of the antigens are characteristic of the spore, others are held in common with the parent vegetative form. There is no evidence that any particular antigen characterizes all endospores. To the contrary, the endospores of different species are antigenically distinguishable. Evidence also exists that differences in the antigens of spores and vegetative cells include polysaccharides as well as proteins (28, 63). These findings mean that the formation of the endospore must be accompanied by synthesis of new organic material. During sporulation, the appearance of the forespore cannot be a mere matter of a separation of a portion of chemically unmodified vegetative cell material. These findings also suggest that in seeking for any chemical differences between the endospores and vegetative cells contributing to their unlike biological natures it will be more productive to think in terms of differences in the properties of individual organic constituents, rather than gross differences in their over-all content of particular kinds of

substances, such as water, lipids, and proteins. In any case studies of antigenic structure have provided the only unquestionable proof of serious differences in the organic chemistry of endospores and vegetative cells.

The endospore as a resting body does not manifest any great metabolic activity. This does not mean that endospores do not possess an active metabolism. To the contrary, a variety of enzymes including catalase and gelatinase, and of respiratory processes have been described (93). Low as it is, the endogenous respiration of moist spores is readily evident and measurable with the Barcroft-Warburg respirometer. The few studies done on the respiration of endospores suggest that they contain a full complement of the same kinds of enzymes and coenzymes found in vegetative cells. But the quantities may be much lower; thus six per cent of the cytochrome and fifty per cent of the hematin content of vegetative cells have been reported to occur in the endospores of *Bacillus subtilis* (53).

While the conditions necessary for the formation of endospores are not well understood and have been much debated, two observations seem to have been made frequently enough with a sufficient variety of species to deserve the status of generalization. These are: (1) the optimum conditions for sporulation are like those for growth of the vegetative form, the permissible variation in the environment being within shorter limits than those for growth, and (2) the sporulation commences after the logarithmic or period of most rapid vegetative growth. These facts are contrary to old teleological ideas and textbook statements postulating harmful conditions in the environment as causes of sporulation. The thought was that since spores were resistant bodies they must have the biological function of carrying the organism through a period of hard times, and thus were formed in response to harmful conditions of environment. No critical research in the recorded literature supports this reasoning.

Actually, the biological role of endospores is unknown. Since one bacterial organism forms only one spore which in turn yields one vegetative organism, the endospore cannot be said to be a device that multiplies the number of individuals of a species. Because spores in higher plants have a role to play in sexual processes or in phenomena of nuclear rearrangement, there has been a stimulus to seeking a similar role for the bacterial endospore. No direct or indirect evidence exists for sexual processes in bacteria involving endospores. Cytological studies of dead stained material have sometimes been claimed to support the idea that endospore formation is an autogamic reproductive process. But present evidence does not permit the acceptance of autogamy, or the fusion of nuclei of the same cell, as the underlying phenomenon associated with sporulation. The descriptions of autogamic processes by various authors do not agree in detail (51). Definitive proof has not always been furnished that the objects called nuclei are not inclusions. Nor do genetic studies lend any support. Thus ultraviolet and x-ray irradiations affect both endospores and parent vegetative forms in the same way as far as the rate and phenotypic expression of mutation are concerned. The mutation data have been interpreted to mean that both vegetative cells and endospores are uninucleate and probably haploid (17, 69). In a process of autogamy one would expect to observe some diploid phase.

The most recent suggestion has been to assign a role to the bacterial spore as an agency for the aerial distribution of the species (9). Evidence for this view is poor and entirely speculative. For one objection, both aerobic and anaerobic sporeforming species are not grown directly exposed to the atmosphere. Their opportunities for being swept up by wind currents are completely fortuitous rather than predetermined by their circumstances of growth. They grow in bodies of water, in soil when the moisture content and relative humidity are not favorable to pulverization, in the intestinal tract, and in marine sediments. As a matter of fact, these latter organisms exist at depths of the sea removed for eons of geologic time from any contact with the atmosphere of the earth (109). To rescue the hypothesis, we would have to invent another hypothesis, namely, that marine sporeforming bacteria are descended from terrestrial types. But it is poor scientific method to support one hypothesis by inventing another and unverifiable one. In this particular case, the hypothesis of a terrestrial ancestry for marine species in the absence of any evidence is totally unacceptable. As a rule, evolution has proceeded from marine forms toward terrestrial forms. There is no reason for considering marine sporeforming bacteria an exception.

It has also been claimed that endospore adaptation to aerial distribution is shown by their reduction in size and weight (9). Thus it is stated that spores are "considerably reduced in size for the sake of lightness and, therefore, concentrated. It is this concentration of the protein by the elimination of water . . . ". But this is a misquotation of fact. In spite of persistent beliefs to the contrary, the chemical analyses available in the scientific literature do not indicate a significant difference in the total water content of spores and vegetative cells. Bacterial spores uniformly show a greater specific gravity or density than homologous vegetative bacilli.[1] The rate of fall of particles through fluids such as water and air is governed by Stoke's law. This law states that the terminal velocity of fall of a spherical object through a fluid is equal to

$$\frac{2r^2(d_p - d_f)}{9\eta}\, g,$$

where r is the radius of the sphere, d_p the density of the particle, d_f the density of the fluid, η the viscosity of the fluid, and g the gravity constant for acceleration. It is not the absolute weight that is determining in the capacity of an object to remain suspended in air, but the surface area of the object relative to its total volume, and the difference in density of the object and the air. Some simple calculations show that the bacterial spore is only slightly more able to float in quiet air than vegetative cells (table 1.1). When one recalls that natural objects much larger and denser than vegetative bacilli, such as pollen grains up to 100 μ in diameter, and fungus spores 10 to 12 μ in diameter with

[1] It is quite possible for biological objects with the same water content to have dissimilar specific gravities greater than 1.0. This would depend on the relative proportions of the cellular constituents with specific gravities greater than 1.0, namely, proteins; carbohydrates; nucleic acids; mineral salts. Possibly the greater density of spores than vegetative cells may be indicative of a relatively higher nucleic acid content, sp gr 2.0.

specific gravities of up to 1.4 are air-borne with ease (16), the relative advantage of bacterial spores in aerial flight over somewhat larger and less dense vegetative bacilli seems dubious indeed. Then again, in the natural situation, bacteria probably frequently do not float in the air as discrete particles, but rather adhere to soil, dust, and water particles of sizes larger than themselves. In these cases the surface area and density characteristics of the transporting particles would seem of greater significance than the properties of the smaller bacilli and spores.

From this resumé of the biological roles which have been suggested for the bacterial endospore, it is evident that the need remains for new ideas, especially for hypotheses which can be treated experimentally. The presentation of purely

TABLE 1.1

Ratios of the terminal velocities of various natural objects predicted from Stoke's Law

	SIZE	SPECIFIC GRAVITY	IDEALIZED SHAPE	RATIOS OF TERMINAL VELOCITIES IN AIR	
				Endospore to	Vegetative cell to
Bacillus cereus endospore.....	1.0 x 1.5 μ	1.2	Cylinder		
B. cereus vegetative cell.....	1 x 4 μ	1.1	Cylinder	3.7	
Aminitopsis vaginata spores.....	9.6 μ	1.02	Sphere	50	14
Timothy grass pollen.....	35 μ	1.0	Sphere	653	178

Bacillus cereus was chosen as a soil organism commonly found in air masses over land. The calculations recorded have assumed a viscosity of 0.00018 poises, and a negligible density (0.0012 for dry air at 1 atmosphere pressure and 18 C) for air. In addition, it was assumed that a cylinder would fall through air at a rate equal to that of a sphere of the same surface area.

Buller (16) has found fungus spores to have a tendency to fall with their long axis parallel to the earth's surface, or so as to be oriented with their greatest surface presented to the resistance of the air. If the same holds true of bacteria, vegetative cells with their greater ratio of transverse to longitudinal axis than spores (2.7 times greater in the case of *B. cereus*) would have an advantage in this regard over spores as air-borne objects.

speculative hypotheses would seem to be gratuitous and deserving of the most critical scrutiny. At the moment we are in the unenviable position of having to agree with Cook (20) that some bacteria "form spores because they form spores".

Part II. The Cytology of Sporulation

GEORGES KNAYSI

The cytological changes resulting in the formation of endospores have been highly elusive. The extensive literature on this subject has been recently reviewed by Knaysi (58). Three more papers have appeared since the publication of this review: one by Delaporte (26), another by Bisset (10), and a third by Hunter and DeLamater (51). Delaporte and Bisset relied chiefly or exclusively on staining with Giemsa's solution after hydrolysis with acid, whereas Hunter and DeLamater used a modification of the Feulgen reaction which, however,

does not have the same significance since it involves treatment with Schiff's reagent. Descriptions of the process by the several authors are not entirely similar. There is agreement that the forespore is formed by growth around a single nuclear unit and that extrasporal nuclear units finally disintegrate, but there is no general agreement as to the number of units that disintegrate. Bisset describes a rodlike nucleus arising by division from an autogamic fusion-nucleus; Hunter and DeLamater emphasize the asexual nature of the endospore. There is also disagreement on whether the spore nucleus is peripheral or central. Furthermore, growth of the forespore is not in harmony with cinematographic and other photographic records showing that the forespore makes its appearance fully grown.

More than two years ago the author resumed investigation of the cytology of endospore formation, and after a certain period of confusion finally succeeded in developing a systematic procedure with criteria of such significance that the customary guessing is either greatly reduced or entirely eliminated.

PROCEDURES AND ORGANISM

The process of sporulation may be investigated in two ways: (*a*) by observing the sequence of changes leading to the formation of the endospore in the living cell; (*b*) by reconstructing a sequence of changes from the observation of stained or inactive cells at various stages of the sporulation process. The first method attains a high degree of accuracy when the sequence of events is recorded by cinematography (5), or by serial photography (57), but is limited by the degree with which it can differentiate the various structures involved in spore formation. The accuracy of the second method depends on technique and judgment but is much superior to the first in revealing and differentiating various structures. The two methods are complementary.

In the *study of the living cells* we employed strain C_3 of *Bacillus cereus*. The technique used was similar to that previously used by the author on the same organism (57). Since the microcultures observed must complete their normal development in monocellular layers, one has to use a dilute medium. In the investigations on which this report is based we used a medium consisting of 0.01 g of dry yeast extract, 2 g of agar, and 100 ml of distilled water. The observations were made with the phase microscope, in dark contrast, and recorded on 35 mm, Panatomic-X film with a Contax camera having a special attachment to the microscope.

In some of the preliminary work on *stained cells* we employed strain 13 of *Bacillus agri* (Lamanna, 62), an organism morphologically similar to *Bacillus brevis*. However, most of the work was done with strain C_3 of *B. cereus*. Real progress resulted from the application to the latter organism of the principle of physiological treatment (60). The technique of Hillier, Knaysi and Baker (45) revealed that when the medium supporting the collodion film consists of 0.2 per cent sodium acetate, 0.2 per cent glucose, and 2 per cent of agar in distilled water, the spores of *B. cereus* can germinate, forming a culture in which the normal life cycle of the organism is readily completed. In these cultures, it is

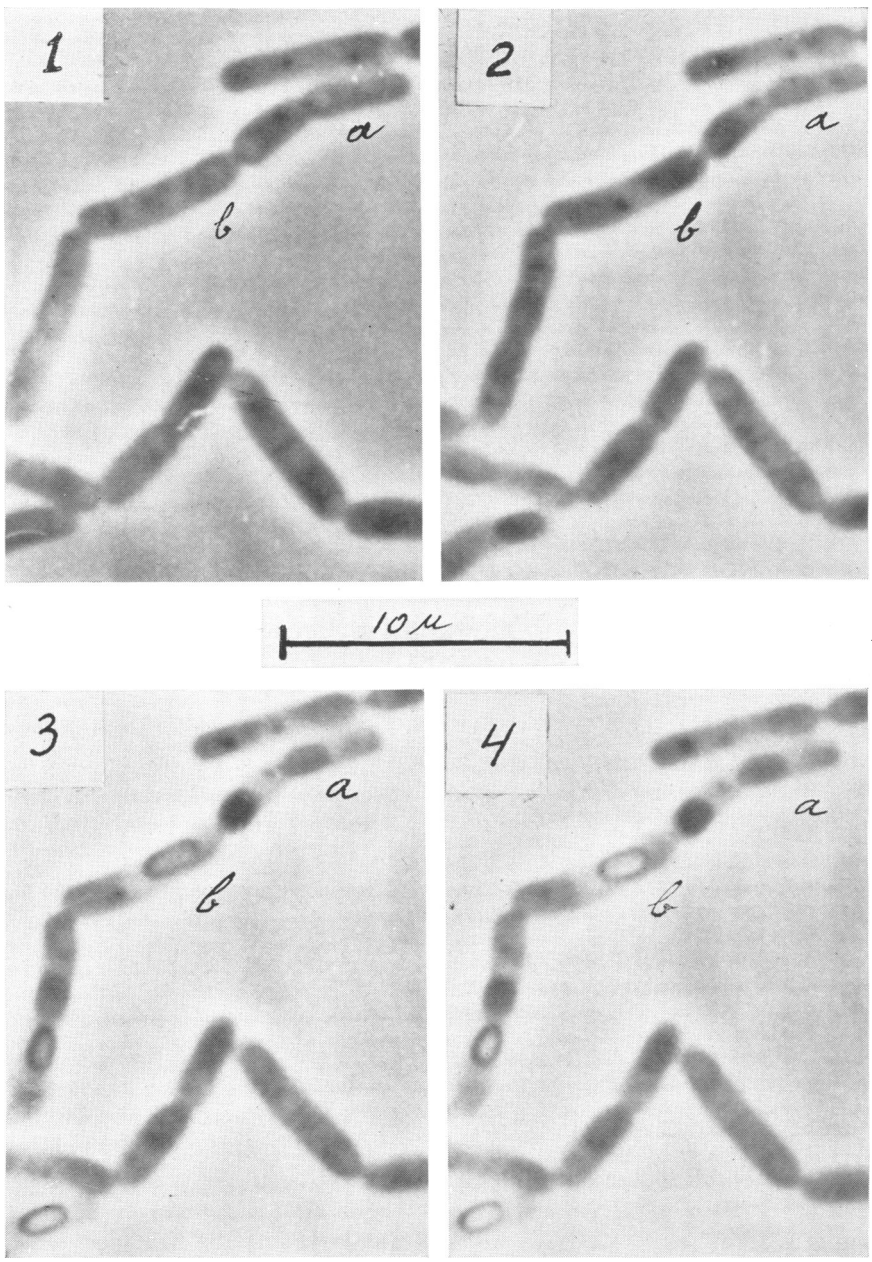
found that in all cells, from germ cell to sporangium, the only structure that stains deeply with methylene blue is the nucleus which stains in two colors and clearly shows its structural organization; all other cellular structures are either faintly stained or not stained at all. Furthermore, in these cells it is possible to distinguish between a vegetative nucleus and a forespore nucleus or forespore. This behavior seems to be characteristic of all strains including var. *mycoides* of *B. cereus* in our collection.

We generally used a 0.1 per cent, aqueous methylene blue solution of pH 3.5, although similar results may be obtained over wide ranges of pH and concentrations. At pH 8 the cytoplasm begins to stain less faintly, but the internal structure can still be clearly seen. The fixed preparation is mounted in a thin film of methylene blue solution under cover glass, sealed with vaspar (a mixture of equal parts of vaseline and paraffin), and observed with the microscope. We used a combination of 90 × apochromatic objective, N. A. = 1.40, and 20 × compensating ocular. Wratten filters, single or in combination, were often used to increase contrast and sharpness of the images. In these preparations the lipoid inclusions remain colorless but may be stained with Sudan III without decolorizing the nucleus. The observations were recorded photographically as with living cells, i.e., on 35 mm, Panatomic-X film in a Contax camera. A number of pictures was also taken on Kodachrome film.

OBSERVATIONS

The preparatory changes are similar to those described in the same organism by Knaysi (57). When a culture is developing in an environment where endospores may be readily formed, the culture enters the sporulation stage soon after vegetative growth and reproduction have practically ceased. This stage is initiated by the appearance in the cytoplasm of a number of minute lipoid inclusions often with a lively Brownian motion. These inclusions gradually coalesce forming fewer but larger inclusions. They appear dark when observed in dark contrast with the phase microscope and are not as conspicuous as they appear with the ordinary microscope.

Formation of the forespore is initiated by the appearance, close at the pole of a potential sporangium, of a light body surrounded by dark rim with a diffuse, somewhat irregular margin. At first this light body is somewhat flat but grows slightly in length and appears round even at a magnification of 1,500. At a much higher magnification, however, it may be seen that the outline of the light body is in reality parallelogrammic, and that the body itself consists of one pair, or sometimes two pairs, of smaller, parallel light bodies, rodlike or having the shape of a dumbbell. Indeed, it can be readily seen that the light body has the structure typical of the bacterial nucleus (figures 2.1 to 2.4). The lipoid inclusions and any other nuclei that may be present migrate toward the opposite end of the young sporangium (figures 2.1 and 2.2). A comparison of cell *a* in figures 2.1 and 2.2 shows that the presence of two pairs of parallel, chromosomelike bodies results from an increase in the size of the forespore nucleus and division of the chromosomelike bodies.



FIGS. 2.1 to 2.4. Strain C_3 of *Bacillus cereus*. A microcolony at different ages, showing stages in the development of the endospore. *1*, 21 hr + 50 min; *2*, 24 hr; *3*, 25 hr; *4*, 25 hr + 20 min. Room temperature, 27 to 30 C. Medium = 0.01 g yeast extract powder + 2 g Bacto agar + 100 ml distilled water. Note, particularly in *1b*, that the forespore nucleus contains two, chromosomelike, parallel light bodies. Compare *1a* with *2a* and note the increase in size and the cross division of the forespore nucleus in *2a*. Note also in *3* and *4* that the light chromosomelike bodies remain perceptible for some time within the forespore. All four figures have the same scale.

The dark rim which surrounds the light body, henceforth called the forespore nucleus, probably represents the nuclear membrane enveloped by dense material synthesized by the nucleus or deposited around it. The dense envelope gradually increases in thickness and optical density, obscuring the structure of the forespore nucleus. In some cases the parallel, chromosomelike bodies within the forespore nucleus may still be perceived after 2 or 3 hours, but finally the forespore becomes homogeneously black as it attains its maximum, or nearly maximum, dimensions.

Growth of the forespore, i.e., the increase in the thickness of the dense envelope, takes place chiefly in the direction of the long axis of the sporangium. It is not clear whether the lipoid inclusions are pushed away by the growing forespore or are moved by cytoplasmic currents. The light body or forespore nucleus appears soon after migration of the inclusions begins, but the inclusions are seldom in contact with the forespore; there is usually a homogeneous zone separating inclusions from forespore. This seems to suggest an action of currents, but evidence from the study of stained material indicates that the mechanism of migration may not be immutable.

The free surface of the fully grown forespore, near the middle of the sporangium, is at first flat or irregular (figure 2.2) but soon assumes the characteristic, convex form. Frequently, the free surface is concave, apparently because the synthesis or deposition of the dark substance goes faster in the ectoplasmic layer than in the axial column. In these cases, the change from a concave to a convex meniscus is accompanied by reduction in length. In the case of one forespore selected at random the reduction is from 2.50 to 1.75 μ. Before the forespore attains its maximum size, it usually undergoes a change of position, moving toward the middle of the sporangium (cf. figures 2.2 to 2.4). The forespore stage ends with the formation of the spore coats.

The transition from forespore to spore is marked by a differentiation of the uniformly dark forespore into a shiny, not a transparent, core and a dark rim. At first the inner margin of the rim is wavy and somewhat diffuse, but in a little more than 30 minutes the rim becomes of uniform thickness and with clear-cut, smooth margins. In the completed spore the shiny core is usually 0.6 to 0.8 μ in width and 1.1 to 1.6 μ in length. The entire spore is 1.1 to 1.2 μ in width and 1.5 to 2.1 μ in length. The thickness of the rim is 0.15 to 1.25 μ. The form of the spore is mostly ellipsoidal or oval.

Stained Microcolonies. The vegetative nucleus appears in two colors: the chromatin stains very dark purple and the nucleoplasm, also possibly the nuclear membrane, pink. The cytoplasm stains faint blue and the lipoid inclusions remain colorless. The cytoplasmic membrane usually stains slightly darker than the cytoplasm. The cell wall and slime layer both appear light purple. As soon as a forespore is initiated, the initiating nucleus changes in its staining properties. Instead of staining in two colors like a vegetative nucleus, the forespore nucleus stains light blue. We believe that the change in color is not due to a change in the chemical composition of the nucleus, but to the synthesis or deposition of a blue-staining substance around the nucleus. This substance

is superimposed on the nucleus and soon masks its structure. In a very early stage of forespore development the typical structure of the nucleus can still be made out, and the red color of the nucleoplasm can be revealed by high focusing.

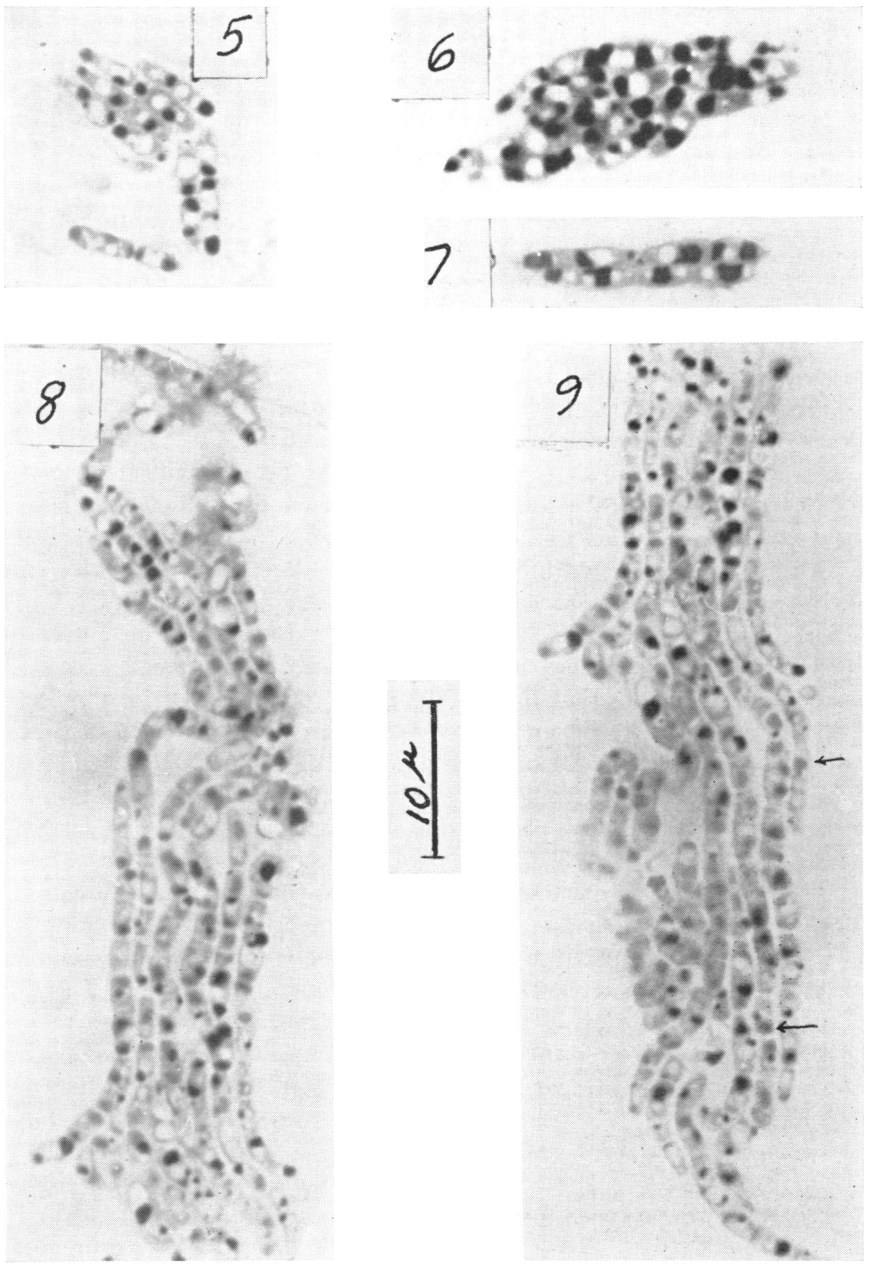
A potential sporangium may contain one, two, or several nuclei often of widely different dimensions. The nucleus which is to initiate a forespore is almost always located at one of the cell ends. In two contiguous, potential sporangia the potential forespore nuclei are often located at the contiguous ends of the sporangia. Examples in which the forespore nucleus is not terminal are rare. The blue-staining substance at first obscures the structure of the forespore nucleus, and as more of it is formed, the nucleus is completely masked as the dimensions of the forespore gradually increase, particularly in the direction of the long axis of the sporangium. The forespore finally appears as an ellipsoidal or oblong body near the middle of the cell and often surrounded by an unstained zone. Any lipoid inclusions and nonfunctional nuclei that were in the spore side of the sporangium are now in the other side, either because they were carried by cytoplasmic currents or pushed away by the developing forespore. However, the evidence here favors the pushing away by the forespore, and one sees here and there a nonfunctional nucleus at the surface of a forespore (figure 2.9). Here cytoplasmic currents are probably minimized by a very slow cellular activity and the presence of very large lipoid inclusions (figures 2.8 and 2.9).

The transition from forespore to endospore apparently does not involve any visible change other than the formation of the coats and the usual dimensional changes. Mounted in the dye solution, a free spore shows a purple rim surrounding a refractile, colorless core. The stained rim is, apparently, the outermost coat. The large volumes of lipoid inclusions formed under the conditions of our experiments and the clear-cut distinction between them, regardless of size, and the nonfunctional nuclei by the technique of double staining with methylene blue and Sudan III permit the conclusion that the volume of the lipoid inclusions decreases considerably during the process of sporulation. The endospore, nonfunctional nuclei if present, and any residual lipoid inclusions are liberated by disintegration of the sporangium.

DISCUSSION

In the investigations here reported one finds, for the first time, almost complete harmony between the process of sporulation as observed in the living cell and that reconstructed from the study of stained material. This was made possible, on the one hand, by the use of the phase microscope which brought out changes not previously observed in the living cell and, on the other hand, by a technique of growing and staining that preserved the positional relationships of the cells and permitted observation of their structural differences and cytochemical changes at all cultural stages. Indeed, the techniques of growing and staining the cells eliminated guesswork and made a systematic study of the process possible.

It is generally believed that the endospore is an asexual spore of high resist-



FIGS. 2.5 to 2.9. Strain C_3 of *Bacillus cereus*. Collodion cultures on the medium, Na acetate, 0.2 g + glucose, 0.2 g + agar, 2 g + distilled water, 100 ml. Spore inocula were incubated at 27 to 30 C. Fixed with ethyl alcohol for 2 min and mounted in 0.1%, aqueous methylene blue solution of pH 3.5. *5*, one day; *6*, one day + 23 hr; *7*, two days + 1 hr; *8* and *9* represent contiguous parts of a colony 3 days old. Note the considerable increase of the black nuclei in *6* and *7* as compared to *5* and also to *8* and *9*. Note, however, that the number of nuclei per cell does not change. In *8* and *9* the gray bodies are forespores at various stages of development. In all 5 figures the white bodies are lipoid inclusions. In *9*, the arrow points to a minute nucleus occluded in a young forespore. All five figures have the same scale.

ance, formed to carry the strain through adverse conditions. Several investigators, however, attributed to the endospore a sexual nature and claimed the observation of nuclear fusion and reduction division before its formation. The literature on this subject was reviewed by Knaysi in 1948 (58). Since the publication of this review, sexuality of the endospore has been affirmed by Bisset (10), but without bringing new evidence. It is natural, therefore, that in the course of this investigation we remained alert to any evidence of sexuality. Since the only nucleus we generally recognized with certainty in the living cell was the one which initiated the forespore, our observations had, perforce, to be restricted to stained material. We noted that the young germ cell shows one, two, or sometimes three nuclei of moderate or small sizes. We did not always find a correlation between the stage of development of the germ cell and the number of nuclei it contains. As the germ cell grows and multiplies, in about one day the progeny consists of cells containing from one to several nuclei each mostly 2 and 1. The nuclei of the same cell as well as those of different cells may be much more different in size than those of the germ cells (figures 2.5 and 2.6). In particular one observes in certain cells large nuclei generally in terminal positions, and many of these nuclei appear as if in the state of incomplete division or as if at different stages in the fusion process. In cultures 2 days old, the picture is similar except that the large nuclei become even larger, more widely distributed among the cells, and more actively dividing (figure 2.7). It may be noted that, regardless of the size of the nuclei, their number per cell does not change. Depending on the temperature of incubation, forespore formation begins in cultures from 3 to 5 days old. At this stage, the large nuclei are less widely distributed in the culture than they were between the second and third days (figures 2.8 and 2.9). The young sporangium does not often contain a very large nucleus, but even when it does the forespore is never initiated by one of these nuclei but by one of moderate to small size. These observations do not favor sexuality of the endospore, and we do not expect the final solution of this problem to result from the study of nonliving material. Nuclear fusion may well take place in vegetative cells of sporeformers, but this would not prove sexuality of the endospore, since nuclear fusion has been observed in bacteria that do not form spores.

SUMMARY

The cytological processes involved in endospore formation in strain C_3 of *Bacillus cereus* were studied both on living cells, using the phase microscope, and on stained colonies by a novel technique based on the work of Knaysi and Baker (60). Almost complete harmony was found between the results of the two studies. A potential sporangium may contain one to several nuclei. The forespore is initiated by a terminal nucleus of moderate size. Dense material with characteristic staining property is deposited around this nucleus, forming an envelope which grows to the maximum size of the forespore. Presporal inclusions and other nuclei that may be present in the fertile part of the sporangium move, or are pushed, to the sterile part. Occasionally a small nucleus may stick

to the forespore and become enclosed in the endospore. As far as the forespore nucleus remains visible, it continues to occupy a central position within the forespore. Sometimes the pair of chromosomelike bodies it contains divide forming two pairs. Before the forespore attains maximum size it moves toward the median part of the sporangium. A highly refringent coat is formed within the boundary of the forespore, leaving a peripheral layer which becomes the outer coat of the spore. The problem of sexuality of the endospore is discussed.

PART III. SOME PHYSIOLOGICAL ASPECTS OF BACTERIAL SPORE FORMATION AND SPORE GERMINATION

E. STATEN WYNNE

SPORE FORMATION

The literature on physiological features of endospore formation has been reviewed by several authors (14, 20, 55, 58, 98). Though there would seem to be at present a fairly general agreement with the view of Knaysi (58) that sporulation is a normal process, a divergence of opinion exists as to the fundamental nature of this process and the underlying conditions leading to its expression. Knaysi has concluded that endospores are formed by healthy cells facing starvation, not only in all strongly aerobic members of the genus *Bacillus*, but probably also in other *Bacillus* species and members of the genus *Clostridium* as well. However, the role of starvation has been questioned recently (36, 38). According to Foster and Heiligman (38), the process of spore formation may be considered as a sequence of integrated biochemical reactions which are independent of vegetative growth and may be interrupted at certain susceptible stages. A third recent view expressed by Schmidt (88) holds that sporulation is a function both of the environment and of "cellular factors" determining reaction to environment.

Although we are in essential agreement with the conclusions of Foster and Heiligman and of Schmidt, we wish to call attention to a much older concept of spore formation which we believe is of value in consideration of the process of sporulation and the great mass of investigation concerning it.

BEHRING'S HYPOTHESIS

In 1889 E. Behring (6, 7) suggested that spore formation be considered as an intermediate stage in normal development which may be partially or completely inhibited by any partial physiological damage short of total prevention of growth. It is interesting to examine Behring's concept in the light of reports on the effect of diverse environmental factors on sporulation, with special reference to recent work on antisporulation factors and so-called "stimulatory" factors.

General environmental factors

Many data concerned with a variety of environmental effects attest to the essential correctness of Behring's hypothesis. Behring himself reported the sub-

sequently often confirmed finding that pH values not appreciably affecting vegetative multiplication completely prevented formation of spores. This worker also found that several growth inhibitors prevented sporulation at concentrations roughly one half of those at which growth ceased. Several investigators (24, 68, 72, 79, 80, 89) have reported that temperature ranges for sporulation are generally narrower than those for vegetative growth. Oxygen tensions unfavorable for spore formation have allowed good vegetative development (65, 102). Similar findings have been reported for such factors as pressure (68), disinfectants (87), growth products of other organisms (13), surface tension (103), and salts (65, 103).

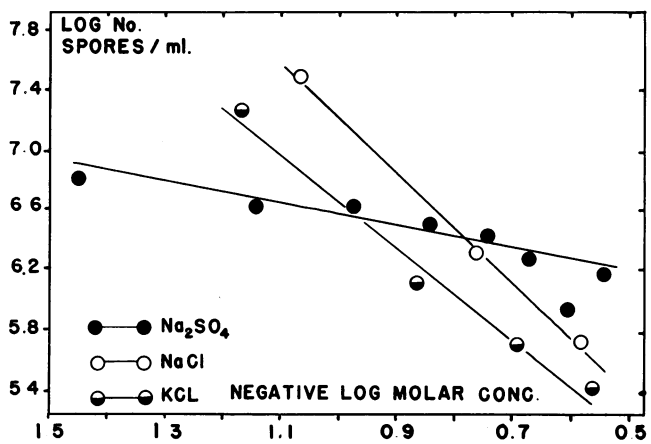


FIG. 3.1. Effect of salts on sporulation of *Clostridium botulinum* 62A. From (103).

With 3 salts tested, an apparent exponential relationship has been noted between concentration and absolute number of spores (figure 3.1). It was suggested (103) that this relationship may be expressed mathematically by the equation

$$C^n S = K \qquad (1)$$

where C = molar (or ionic) concentration of salt tested
n = dilution coefficient — a constant for the particular salt
S = no. spores/ml, and
K = a constant.

Antisporulation factors

Students in bacteriology have often been taught that spore formation occurs when nutritive substances are depleted, and that sporulation, therefore, is poor in concentrated media. However, recent investigations have indicated that the presence of antisporulation factors may account for poor sporulation in concentrated media. It was reported in 1942 by Roberts and Baldwin (86) that peptone concentrations allowing growth but not sporulation gave good yields of spores following adsorption with charcoal. Recently Foster *et al.* (36) found that treat-

ment of complex media with charcoal or soluble starch gave substantial increases in growth and percentage of spores in *Bacillus larvae*, an organism in which difficulties in securing appreciable sporulation have been commonly encountered. Nutrient agar, which allowed some growth but no spores, yielded 50 per cent spores after the charcoal treatment. Evidence has been presented by Hardwick *et al.* (41) that at least part of the antisporulation activity of complex organic media for several aerobic species resides in certain nonvolatile saturated fatty acids. It should be noted that while sporulation was completely inhibited by 50 μg/ml of a nonvolatile fatty acid fraction from peptone, 150 μg/ml were required to inhibit completely vegetative growth.

Experiments frequently cited in support of theories of sporogenesis involving starvation can probably be explained on the basis of a reduction in the concentration of antisporulation factors rather than depletion of nutrients. One example is the finding of Buchner (15) that sporulation in *Bacillus anthracis* occurred faster when vegetative cells were incubated in distilled water than in the culture from which they were transferred. A second is the report of Bongert (13) that *B. anthracis*, which did not sporulate in undiluted blood, gave excellent spore formation in blood which was greatly diluted with water. Furthermore, increasing the concentration of nutrients does not invariably lessen the percentage of spores, or vice versa. Brunstetter and Magoon (14) reported that 3 of the *Bacillus* species tested showed an increase in the percentage of spores as the concentration of peptone increased. It was found recently (103) that a 4-fold dilution of a brain heart infusion medium did not result in an appreciable increase in sporulation. Although concentrations of the medium above normal did markedly reduce percentage of spores, much of this effect could be ascribed to the increased NaCl concentrations.

The point we wish to emphasize is that the effect of antisporulation factors forms an important illustration of Behring's hypothesis.

Nutritional factors

Mellon (71) reported that sporulation in one species occurred earlier and more abundantly when a filtrate of a culture of a symbiont strain was added. Comparatively few studies have been carried out regarding chemically identified nutritional factors involved in spore formation. The view was expressed by Foster and Heiligman (38) that nutritional factors favoring sporulation may be considered to do so by providing the proper enzyme balance and do not necessarily "stimulate" the onset of spore formation *per se*. This concept is obviously in accord with Behring's hypothesis.

Various ions have been reported to have a favorable effect on sporulation. Leifson (65) reported that phosphate and ammonium, and to some extent sulfate, ions increased spore yields in *Clostridium botulinum*. Univalent cations such as sodium, potassium, and lithium favorably influenced sporulation in *Bacillus* species in the studies of Fabian and Bryan (33). Recently Foster and Heiligman (37) found that addition of potassium to an N-Z case medium resulted in in-

creases in spore percentages in *Bacillus cereus* of more than 10-fold, while the effect on vegetative growth was slight.

Almost no investigations of amino acid requirement have been made. Of 19 amino acids tested for ability to reverse the toxicity of DL-alanine toward spore formation in a strain of *B. cereus*, Foster and Heiligman (38) found that only leucine and isoleucine were active, and these had relatively small effects. The beneficial action of leucine on sporulation in *B. cereus* has been confirmed by Williams and Harper (99). Blair (11) reported that omission of methionine from a synthetic medium suppressed spore formation in *C. botulinum*, but it should be noted that vegetative development was greatly lessened also. Furthermore, lysine was found to substitute for arginine with approximately 50 per cent efficiency for vegetative cell yield, but no spores were noted. A somewhat similar relationship was found when an increased tyrosine concentration was used to substitute for phenylalanine. These findings would seem to indicate that any amino acid deficiency affecting growth is apt to affect sporulation more strongly, a generalization quite in accord with Behring's hypothesis.

Virtually no confirmed fundamental knowledge exists concerning the role of growth factors in sporulation. Hayward (42) noted a slight beneficial effect of inositol for a strain of *B. subtilis*, and Williams and Harper have noted such an effect with *p*-aminobenzoic acid on two strains of *B. cereus*.

All the cited facts on sporulation are compatible with Behring's hypothesis, and likewise with the view of Foster and Heiligman that sporulation involves a balanced integrated series of enzymatic reactions.

SPORE GERMINATION

Criteria of germination

Most of the older studies on spore germination employed the appearance of visible turbidity as a criterion of germination. Such an approach obviously yields no quantitative information but can be of value in establishing that some germination has or has not occurred, provided that conditions are such that subsequently vegetative cells can develop. However, several workers have clearly shown that germination may occur under a variety of conditions preventing significant vegetative multiplication (19, 52, 56, 60, 61, 107).

Direct microscopic counts have been employed, but such techniques are laborious and not readily adapted to anaerobes. Furthermore, it is difficult to establish a microscopic criterion of germination.

Development of heat lability has also been employed by several workers (32, 34, 47, 96, 104). It has been claimed that acquisition of the heat-labile state may precede rupture of the spore wall (22) and that certain morphological changes may occur prior to loss of heat stability (61). However, the definite reproducible end point attainable makes this criterion of germination a workable one for quantitative studies of germination. It should be pointed out, however, that heat resistance of spores of species examined by this technique should be relatively great.

Quantitative methods of study

Various quantitative methods of study of spore germination have been developed using heat lability as a criterion of germination. Recent methods have generally employed heating the spores before inoculation, in view of the recognized beneficial effect of "heat activation" on germination (32). After incubation in a suitable medium, a second heating is employed to destroy vegetative cells. Differences in spore counts made by plating techniques before and after incubation are used for evaluation of the amount of germination. We have preferred comparisons of per cents of spores germinated rather than the numbers of residual spores remaining, since with this method of expressing results there is less likelihood of attaching a false significance to small deviations.

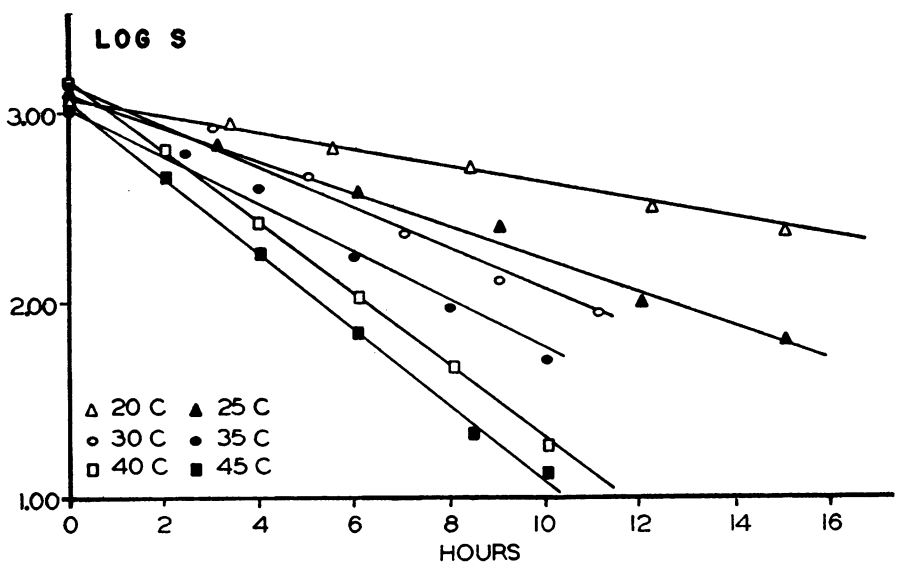


FIG. 3.2. Germination curves of putrefactive anaerobe no. 3679. From (70).

Certain other precautions must be taken in using such a method. The well known phenomenon of dormancy, resulting in delayed germination of some spores, particularly after heat treatment, must not occur in sufficient degree to be a practical obstacle. Incorporation of soluble starch in the germination medium seems to be effective in preventing, or at least minimizing, dormancy with *C. botulinum* (77). Furthermore, in testing substances for possible inhibition of germination it must be shown that amounts of such materials remaining after dilutions do not adversely affect development of colonies in the plating media. Likewise, possible sporicidal effects must be ruled out. Finally, the incubation period must be such that resporulation is not appreciable.

With proper precautions, quantitative studies of various types may be carried out. When the logarithms of residual spores are plotted against time, it has been found that a straight line is obtained for germination in *C. botulinum* and putre-

factive anaerobe no. 3679 (70, 105). Representative data for the latter organism are shown in figure 3.2. Germination apparently follows the kinetics of a first order reaction which may be written as

$$0.434\,k = \frac{1}{t}\log\frac{I}{(I - G)} \tag{2}$$

where k = the velocity constant of germination
t = time elapsing since beginning of germination (t_0)
I = no. spores per ml at beginning of germination
G = germinated spores at time t.

If we let $I - G = S$ (residual spores at any time t), equation (2) may be written

$$\log (I - G) = \log S = -0.434\,kt + \log I \tag{3}$$

and

$$k = -m/0.434 \tag{4}$$

where m is the slope of the line obtained by plotting log S vs. t, as in figure 3.2.

TABLE 3.1

Velocity constants of germination for putrefactive anaerobe no. 3679

TEMPERATURE, C	k	TEMPERATURE, C	k
20.0	0.11	34.8	0.32
20.0	0.11	35.0	0.32
25.0	0.18	39.9	0.44
26.0	0.20	40.0	0.45
30.0	0.27	45.0	0.45
30.1	0.28	46.0	0.46

Values of m have been determined by the method of least squares, and corresponding values of k for putrefactive anaerobe no. 3679 are shown in table 3.1.

Variation of equilibrium constants of biological processes with temperature has been expressed by the Arrhenius equation

$$\mathrm{d} \ln k \,/\, \mathrm{d}\, T = \mu/RT^2 \tag{5}$$

where k = velocity constant of limiting reaction
T = absolute temperature
R = gas constant = 1.99 calories per degree per mole
μ = temperature characteristic

From the integrated form

$$\log k = \frac{-\mu}{2.3R}\left(\frac{1}{T}\right) + C \tag{6}$$

it is possible to determine μ, the temperature characteristic, by plotting log k vs. $1/T$ and determining the slope m' by the method of least squares or graphically, since

$$m' = -\mu/2.3R = -\mu/4.57, \text{ and}$$
$$\mu = -4.57\ m'. \tag{7}$$

In figure 3.3 is shown a plot of log k vs. $1/T$ for spore germination in putrefactive anaerobe no. 3679. The Arrhenius relationship was apparently followed

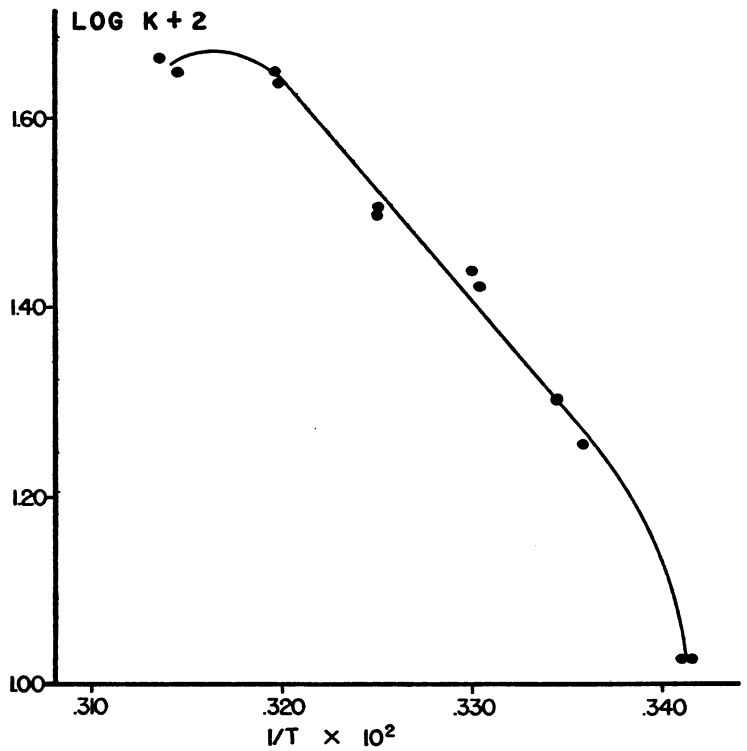


FIG. 3.3. Variation in the rate of germination of putrefactive anaerobe no. 3679 with temperature. From (70).

over a temperature range of 25–40 C, and the value of μ was roughly 10,000 calories.

The lack of effect of penicillin on germination of spores of 3 *Clostridium* species, as shown in table 3.2, constitutes another application of the quantitative method (107). Penicillin was destroyed by the action of penicillinase before platings for counts were made. Appropriate controls showed the lack of a sporicidal effect under conditions preventing germination. Similarly, no inhibition of germination of spores of 3 *Clostridium* species has been observed with streptomycin (19).

For maximum sensitivity in testing inhibitory substances incubation should be such that germination in controls is essentially complete. In table 3.3 the inhibitory effect of oleic acid is shown (39).

TABLE 3.2

Germination in the presence of 1,000 units of penicillin per ml

SPECIES	INCUBATION, HOURS	PENICILLIN	RESIDUAL SPORES	GERMINATED SPORES	PER CENT GERMINATION
C. botulinum, 62A	0	−	480	—	—
	38	−	5	475	99.0
	38	+	10	470	98.0
C. perfringens	0	−	1,750	—	—
	16	−	27	1,723	98.5
	16	+	16	1,734	99.1
Putrefactive anaerobe 3679	0	−	1,960	—	—
	16	−	14	1,946	99.3
	16	+	12	1,948	99.4

TABLE 3.3

Inhibition of germination of C. botulinum spores by oleic acid

OLEIC ACID	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION	INHIBITION
μg/ml	*hours*			%	%
—	0	530	—	—	—
0	24	120	410	77	—
0.1	24	240	290	55	29
1.0	24	400	130	25	68
10.0	24	520	10	2	97

TABLE 3.4

Effect of CO_2 and oxalacetate on germination of spores of C. botulinum in a complex medium

CO_2 IN ATMOSPHERE	OAA 10^{-3} M	INCUBATION	EXHAUSTION PERIOD	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
%		*hours*	*minutes*			%
—	—	0	—	520	—	—
0	0	23	30	470	50	10
0	+	23	30	35	485	93
0	+	23	270	65	455	88
1	0	23	30	21	500	96

On the other hand, maximum sensitivity in testing stimulatory factors is afforded by an incubation time such that germination is relatively slight or negligible in controls. The effect of CO_2 and oxalacetate affords an illustration of this principle (table 3.4). It will be noted that 10^{-3} M oxalacetate essentially

replaced CO_2, even when the vacuum desiccator containing the tubes was exhausted for 270 minutes following the introduction of oxalacetate. There is evidence that the effect of oxalacetate is not due to CO_2 liberated (106).

In synthetic media, only 60 of 560 *C. botulinum* spores appeared to germinate in an incubation time of 17 days; this apparent 11 per cent germination probably did not exceed the experimental error of counting, and no turbidity appeared. In contrast, 340 of 560 *C. botulinum* spores germinated in 5 days with 1 per cent CO_2 in the atmosphere. Carbon dioxide could not be by-passed by oxalacetate or a mixture of tricarboxylic acid cycle intermediates in the synthetic medium but was readily replaced by 0.1 per cent yeast extract (table 3.5). That CO_2 can be by-passed by unknown substances in complex supplements was also reported by Lwoff and Monod (66) for *Escherichia coli*.

TABLE 3.5

By-passing the CO_2 requirement of C. botulinum spores in a synthetic medium with yeast extract

CO_2 IN ATMOSPHERE	YEAST EXTRACT	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
%	%	*days*			%
—	—	0	450	—	—
0	0	15	425	25	6
1	0	5	160	290	64
0	0.1	2	120	330	73
0	1	2	45	405	90
1	1	1	15	435	97

Nutritional factors in spore germination

Very little work has been done on the nutrition of spore germination. Knaysi (60) reported that spores of a strain of *Bacillus mycoides* underwent germination in glucose but not in phosphate or lactose solutions. Hills (48, 49) has recently shown that although slow germination of *B. anthracis* occurred in phosphate buffer a marked stimulation of germination occurred when a mixture of adenosine, L-tyrosine, and L-alanine was added. The effect of L-alanine was not replaceable by related compounds and was strongly inhibited by the D-isomer (46). Requirements of other *Bacillus* species tested varied with respect to tyrosine and adenosine, but all required L-alanine for appreciable germination.

A concept of spore germination

The view has been expressed by Knaysi (58) that germination represents a process involving growth in an environment rich in nitrogen and partly protected from the external medium, usually by a double coat. Factors affecting growth would also be expected to affect germination, with due allowance for the dense coat and rich nitrogen supply. Recent findings have led us to the formulation of an alternative concept of spore germination as a process fundamentally different from subsequent vegetative development. In this connection the following considerations may be noted:

(1) According to Daranyi (24) spore germination is more exacting with respect

to environmental conditions than vegetative growth of the same species. As examples of such conditions, he mentions pH and temperature. Temperature maxima and minima for growth and germination have often been reported to differ. Blau (12) found that 4 species of *Bacillus* had higher maxima for growth than germination, while the reverse was true with 2 other species. It was concluded by Holzmueller (50) from a study of 9 *Bacillus* strains that temperature ranges for germination were narrower than those for growth. It is not apparent how such facts may be explained solely on the basis of the presence of a dense spore coat.

(2) Daranyi reported that germination is more easily prevented by antiseptics than vegetative growth. This finding is difficult to explain on the basis of permeability differences, since one might expect a lower permeability of spores than cells.

(3) Similarly, C_{18} unsaturated fatty acids have been found highly inhibitory to germination of spores of *C. botulinum*, whereas vegetative cell development was relatively quite resistant (39).

(4) On the other hand, it has been found recently (107) that 1,000 units of penicillin per ml had no effect on germination of spores of 3 *Clostridium* species, although vegetative cells were markedly inhibited by 1 unit/ml.

(5) It has been reported recently (107) that up to 98 per cent of the spores of putrefactive anaerobe no. 3679 underwent germination under conditions presumably providing too high an oxidation-reduction potential for vegetative development.

(6) Although adenosine was reported essential for rapid germination of *Bacillus anthracis* by Hills (47), this compound was not stimulatory for vegetative multiplication.

(7) Recently it has been found that temperature changes affected rates of spore germination and vegetative multiplication to different degrees (70).

It is believed that these findings are compatible with the postulation that a fundamental difference exists between the processes of spore germination and vegetative cell development.

Part IV. The Effect of "Culture History" upon Spore Formation on a Standard Test Medium

C. F. SCHMIDT

(Abstract)

Data will be presented to show that "culture history", defined in terms of serial transfers upon media other than nutrient agar, may significantly alter spore formation by obligate thermophilic flat sour organisms when they are transferred back to nutrient agar as a standard test medium. The significance of these results in relation to conclusions drawn concerning the effect of environmental factors upon sporulation and the evidence which these experiments lend to the concept of "cellular factors" having an important effect on the response of an organism to environmental conditions will be discussed.

PART V. RESISTANCE IN BACTERIAL SPORES

HAROLD R. CURRAN

Principal interest in bacterial spores and the basis of their economic significance derive from their extraordinary capacity to survive adverse influences of all kinds. In their capacity to withstand destructive agents they are not equaled by any other living thing. This unique property has led to their adoption as test subjects for sterilization procedures and has determined the commercial method of processing many food and nonfood products.

A few examples will serve to show the magnitude of spore resistance in specific instances. The spores of some flat-sour cultures will withstand steam under pressure at 115 C for over 3 hours, though the vegetative forms of most sporulating and nonsporulating species are killed in a few minutes between 55 and 65 C. Some spores will survive in 4 per cent H_2O_2 for nearly 24 hours, in 10 per cent HCl for almost an hour, in 5 per cent C_6H_5OH for over a week, although death of vegetative cells is almost instantaneous under these conditions. The most resistant spores require for their destruction the application of about 2 million roentgens of cathode or X-ray energy; the corresponding value for vegetative cells is about 500,000–700,000 roentgens. Just how long spores may remain viable under normal environmental conditions is unknown. It is known that anthrax spores dried on silk threads in Koch's laboratory were viable after 60 years and that viable sporeformers have been recovered from canned and hermetically sealed veal after the lapse of 115 years. These examples are rather extreme; some spore cultures are little more resistant than the vegetative form and can be sterilized in a few minutes at temperatures well below boiling. The overwhelming majority are intermediate in their resistance between these extremes.

Resistance varies widely from species to species, to a considerable extent within a species and within a given spore population. It is independent of the luxuriance of growth and of the luxuriance of sporulation. The factors which determine resistance fall conveniently into two categories—those which are intrinsic or inherent in the cell and the extrinsic factors which operate from outside the cell.

The intrinsic factors include species influence or inheritance, and more or less arbitrarily may be included the age of the spores, their specific gravity, relationship to maximum growth temperature and germination characteristics. The spores of *C. botulinum* are most resistant to heat when newly formed (31, 97); in other species, perhaps the majority, maximum resistance is attained after a certain period of maturation. In the flat-sour group and in certain clostridia the spores of greatest density are the most resistant to heat (108). Among the aerobic sporeformers those species with the highest temperature of growth usually have greatest resistance to heat (64). A correlation exists also between the method of exine disposal during germination and resistance; those species which in the process of germination absorb this coat are less resistant than those which shed the exine into the medium (54).

Apart from those factors which are inherent in the cell, various physical and chemical forces in the external environment materially affect the ultimate resistance of the spore crop. These external factors which may enhance or diminish resistance operate in the environment in which the spores are formed, in their subsequent storage surroundings, and in the particular environment in which their resistance is tested, including their subculture environment. Our knowledge concerning the influence of external factors upon resistance is for the most part limited to their relationship to heat resistance.

The nature of the nutrients in the spore-producing medium is significant. With *B. subtilis*, vegetable infusions and isoelectric gelatin yield spores of high thermal resistance, while various digest mediums, excepting casein digest, produce spores of low heat resistance (98). With peptones the brand is more important than its concentration. The effect of utilizable carbohydrates varies with the species. Spores of clostridia formed on pasteurized and sterilized meats and organs are more resistant to heat than those produced on the raw tissues (94). The spores of aerobic bacilli formed in soil and on grains are generally more resistant to heat than those formed on laboratory media. The incorporation in media of certain fatty acids enhances the thermal resistance of *C. botulinum* spores (92); conversely media in which the fatty acid is extracted or adsorbed yield spores of diminished heat resistance. Media deficient in certain metallic ions yield spores of low thermal resistance—those which seem to be of particular significance are the ions of phosphate, magnesium, calcium, and iron (92, 98).

The temperature at which spores are formed is a factor in their resistance. Most workers have recorded maximum resistance at or near the optimum growth temperature; spores of lowered heat resistance are formed at suboptimal growth temperatures (92, 98). After the spores are formed their resistance may increase or decrease, depending on the strain and the particular environmental conditions to which they are exposed.

In the cultivation of bacteria under laboratory conditions, comparatively few external factors are involved, and the action of these is relatively constant. In their natural habitat, a much greater number of environmental agencies are operative and in an ever changing order of succession. Acting to diversify the natural environment are the numerous antagonistic and beneficial influences resulting from the co-existence and growth of a heterogenous microflora. Efforts to evaluate the role of nonnutritional factors in spore resistance have been limited to a few of those most readily controlled and with little attention to their interaction. Slow or rapid drying and alternate wetting and drying increase the thermal resistance of *Clostridium welchii* and *Bacillus* species (43, 98). Spores of *C. botulinum* subjected to rapid drying become slightly less resistant to heat but then remain at this level of resistance for an indefinite period (31). Sand substrates of *B. mycoides* develop their highest thermal resistance under conditions of moderate temperature and humidity; with this species resistance increases with age up to about 60 days and then slowly declines (67). Aqueous suspensions of *Bacillus* species subjected to long continued freezing undergo a

marked reduction in their thermal resistance as compared with spores held the same length of time in the unfrozen state at low temperatures. *C. botulinum* spores stored in their own liquor of growth in the ice box have shown a varied response, some maintaining the same degree of heat resistance for over 4.5 months, others losing a substantial part of their resistance in 2 weeks. *Bacillus* spores exposed for a prolonged period to their own products of growth are much less resistant to heat than those formed in the same batch of medium, washed free of their growth products, and held at similar temperature and period in a dry or wet condition. The conditions under which spores are tested markedly affects their resistance to heat, to chemical substances, and to a lesser extent to radiation. The time required to sterilize a suspension of spores under constant conditions decreases as the temperature increases (31, 95).

The greater the concentration of spores in a medium, the greater is their resistance, i.e., the more heat or the longer is the time required to effect sterilization (31); because of the variation in resistance between different strains and between different crops of the same culture, this statement holds consistently only for dilutions of a given spore crop. The presence of clumps enhances resistance and makes for irregularities in its measurement (31, 98). A longer heating is required to kill spores exposed in open tubes than in similar tubes exhausted and sealed. A major factor affecting heat resistance is the reaction of the medium. The pH of maximum thermal resistance varies with the strain, the medium, the degree of heat, and other factors; usually it is close to the neutral point. However, the pH of maximum heat tolerance of different species may vary from 5.0 to 8.0 (43, 74, 75). In strongly acid or alkaline substrates (below pH 5.0 or above 10.0) thermal resistance is materially reduced (31, 97, 98). Salt in low concentrations increases heat resistance for mesophilic species, both aerobic and anaerobic, but beyond 8 per cent the heat tolerance is diminished (31, 43, 75, 92). In contrast, as little as 1 per cent salt reduces the heat resistance of certain thermophilic types (2). Salts in the concentration used in the curing of meats apparently have little or no effect upon the heat tolerance of the spoilage-significant species, though they may inhibit the germination of the spores after processing.

The influence of moisture in reducing heat resistance is utilized in sterilization procedures. With rare exceptions, bacteriological media containing spores are effectively sterilized by moist steam at 115 C in from 20 to 30 minutes. In dry sterilization as usually practiced, a temperature of 160 to 170 C is maintained for several hours. Some spores may survive dry heating at 300 C for 30 minutes. The presence of organic matter in the test medium when above a threshold amount enhances the resistance of spores against most lethal agents. The effect varies with the nature and concentration of the substance and the organism. At the same pH levels gelatin, peptone, and starch provide maximum heat protection in concentrations from 0.5 to 1 per cent (43, 74, 75). The protective action of sugars has received considerable study. In general, the heat survival of spores in both nutritional and nonnutritional substrates is a function of the concentration in the 15 to 50 per cent range (2, 92, 97). Most substances which have

an antiseptic activity *per se* diminish thermal death time of spores. Heat resistance is markedly increased when spores are suspended in oily materials and the resistance of dry spores suspended in completely anhydrous fat approaches that of dry sterilization. Low concentrations of Ca^{++} or Mg^{++} ions added to spores suspended in phosphate buffer materially lower their thermal resistance (92), although as stated earlier, these ions are of special significance in the production of resistant spores. Recent studies have indicated that the presence of certain vitamins in the test material may alter their resistance to heat; thus, the addition of 100 ppm ascorbic acid to neutral phosphate buffer increases the apparent thermal death time of a *Clostridium* species from 24 to 36 minutes; a synthetic analogue of vitamin K under similar conditions reduces resistance over the control (85). These observations may help to explain variations in thermal death times which have been observed when spores have been heated in extracts which have the same pH but are prepared from different food products. The influence of modifying factors upon heat resistance is demonstrable within a limited range of temperature; when the temperature is sufficiently high, the influence of these factors may be obscured (75).

The conditions of testing the viability of spores after they are exposed to lethal agents greatly affect their apparent resistance. Spores which survive drastic killing influences, whether heat, radiation, or chemicals, are much more exacting in their nutritive requirements than the less resistant individuals which compose the bulk of the population before treatment (22). This necessitates the use of enrichment substances in basal media for the subcultivation of spores previously exposed to destructive agents. The more drastic the treatment, i.e., the fewer the survivors, the greater is the response to fortifying substances. The resistant spores which survive heat tests show also a greater dependence on pH and temperature of incubation and in the case of anaerobes are more dependent on reducing substances in the medium (76, 78, 100). With both aerobes and anaerobes the spores which survive drastic heating are much more sensitive than the less resistant spores to inhibitory substances in the medium (76, 78). Since most of the inhibitory substances present in bacteriological media can be adsorbed on starch (39, 76, 78), this substance is now routinely incorporated in the recovery media in heat processing studies.

In many spoilage-significant strains the majority of the spores are incapable of germination until they have absorbed a certain amount of heat (18, 23). When such cultures are exposed to nonthermal agents, heat activation of the spores before subculture should make for greater accuracy of measurement.

Spores that survive heat or ultraviolet radiation lethal to part of the population are more readily killed by further applications of the same treatment than are untreated spores. When spores are exposed to suitable combinations of heat and ultraviolet radiation, light is found to sensitize some spores to heat, although the reverse sequence indicates that heat has no appreciable light-sensitizing action upon spores. There is little correlation in resistance to different lethal agents—thus the spores of *B. megaterium* are more resistant to ultraviolet radiation than are those of *B. subtilis*, the heat resistance of which is much greater

(29). Similarly, susceptibility to mechanical destruction shows no correlation with thermal resistance. A heat-resistant variant of *B. globigii* was no more resistant to gentian violet, mercuric chloride, and streptomycin than the parent, nonresistant strain, although the former exhibited exhanced resistance to iodine and phenol (25). The thermal resistance of many aerobic species can be increased by the repeated propagation of resistant survivors from heat tests (25, 98), though with anaerobes such efforts have met with little success.

A discussion of resistance is incomplete without some consideration of the mechanism of the process. Although a subject of intriguing interest and practical significance, it must be acknowledged that very little is yet known concerning the basic factors involved. It is at once clear that the process is one of great complexity and involves differing mechanisms, depending on the nature of the lethal agent and the conditions of application. An approach to the problem may be made by considering some of the known facts about spores which seem to have a relationship to resistance in specific circumstances. Lamanna and others (28, 62) have pointed out the antigenic complexity of the bacterial spore —in which some but not all of its antigens are characteristic of the spore. The fact that the spore possesses antigens not found in vegetative cells provides definite evidence of important chemical differences in the composition of organic molecules of spores and vegetative cells which indeed must be presumed to exist and which impart to the spore its extraordinary stability, particularly in respect to denaturation.

The cytoplasm of bacterial spores is relatively rich in diffusely distributed lipoidal material, whereas the membrane also contains fat in combination with protein or carbohydrate. These fat components might be expected to retard the penetration of many substances and must be considered an important factor in their resistance to certain chemical agents. There is some evidence to suggest that lipoidal material in the spore may also contribute to their heat resistance; this consists first, in the fact that rather prolonged extraction of spores with suitable fat solvents such as chloral hydrate and trichlorethylene reduces their heat resistance. Moreover, as stated previously, the presence of certain fatty acids, notably oleic acid, in the sporulating medium contributes to greater heat tolerance of spores produced in such media (92). The removal of the fatty acid by treatment of the spore suspension with chloroform or petroleum ether does not decrease the high resistance of the spores grown in the oleic acid-containing medium, nor does the addition of oleic acid to the spore suspension obtained from the control medium enhance the resistance of these spores, which suggests that the fatty acid may be incorporated in the spores, although its possible breakdown in the cell has not been excluded (92). If, as seems likely, death by moist heat is caused by denaturation of some essential protein, it is plausible to suppose that lipoid material in the spore may protect the peptide linkages from hydrolysis or denaturation and thus prolong their survival.

The presence of a relatively considerable quantity of ribonucleic acid throughout the cytoplasm of the spore undoubtedly protects the protoplasm against such agents as ultraviolet rays and electrons (59).

The long accepted belief that spores contain much less water than vegetative organisms is no longer valid, although important differences seem to exist in the state in which the water exists in the two types of cells. Bound water determinations have revealed that spores contain a far greater proportion of their total water in the bound state than do vegetative cells (40). Water which is bound becomes so intimately associated with the colloids of the cell that it is essentially removed from the liquid state and then will not participate in freezing or chemical reactions. Since death by moist heat is almost certainly a denaturation or coagulation process, less free water might be expected to confer greater resistance against this agent, and limited data show that there is a fairly close correlation between the calculated free water content of spores and the relative thermal resistance of the spores (40). These water relationships for spores and vegetative cells, if generally valid, can help also to explain the greater resistance of spores to certain chemical reagents which by providing less free water reduce the medium of reaction. A study of the mechanism of action of ionizing radiations has revealed that the radiochemical decomposition of water in biological tissue produces oxidizing agents which play an important part in the biological effects produced by these radiations (4). If, in contrast to vegetative cells, most of the water in spores is bound, they might be expected to be less reactive, i.e., more resistant, to such radiations. The same considerations should be factors in the resistance of spores to desiccation and freezing and thawing, a view which finds support in the observation that plants and insects which are unusually resistant to these influences contain a high percentage of bound water.

As previously mentioned, some salts or ions seem to be more significant than others in the mechanism of resistance. A conspicous characteristic of spores is their relatively high concentration of calcium, and thermophilic vegetative organisms contain substantially more calcium than mesophilic vegetative organisms (21). Although there seems to be no direct correlation between calcium content and the degree of heat resistance, it seems reasonable to suppose that the relatively large amounts of this element present in all spores contribute to their resistance. The physiological function of calcium as a cementing agent and its relationship to cellular permeability suggest that it is concerned with the low permeability of spores. The suggestion that the calcium content of spores might be related to their high percentage of bound water has been discounted by Sugiyama (92) on the grounds that large amounts of calcium would decrease rather than increase the amount of water bound by a colloid; also that the amount of water bound by the calcium of spores as judged by theoretical calculations is relatively insignificant in relation to the amount of free water. The same investigator has postulated that calcium ions, by providing a stronger linkage of negatively charged colloidal groups than sodium and other monovalent ions, would confer greater resistance to the unfolding of the peptide chain which would constitute greater resistance to denaturation of the protein.

The unusual resistance of spores to destructive influences may be attributable in some measure to their relatively low salt content. The fact that many proteins

of low ash content resist heat coagulation and alcohol precipitation more effectively than those high in salts has been advanced to support this hypothesis; also it has been observed that spores produced in low-ash media are unusually resistant to heat (98). If, as is now believed, the primary reaction in death by moist heat is that of denaturation followed by coagulation, the effect of salt *per se* on the death process may be negligible. However, it is probable that a decrease in the amount of salts in the cell would decrease the amount of free water, which in turn may increase resistance.

Some investigators have believed that spore resistance is tied up with enzyme survival, possibly the result of a firmer enzyme-protein union (95), which implies the existence of heat-resistant enzyme proteins; however, death of a cell does not necessarily entail destruction of its enzymes. A similar view has been advanced to account for the capacity of thermophiles to grow and metabolize at temperatures above the coagulaging point of typical proteins, but there is no proof that enzymes of spores and vegetative cells are different. A recent study by Allen (1) has shown that the enzymes of actively growing thermophiles are no more resistant to heat than mesophiles.

From the foregoing, it is clear that the physical and chemical basis of resistance is still obscure. The awakened interest and study of spore problems evident in recent years have brought us substantially nearer an understanding of the resistance mechanism and will, we may hope, soon close the gaps in our knowledge of this, one of the most significant and puzzling of biological phenomena.

Part VI. Spores as Reagents for Studies on Chemical Disinfection

MAX LEVINE

For studies on the mechanism of disinfection, the rate of death of bacteria, the time periods required for killing a stipulated proportion of exposed organisms, and environmental factors which affect these criteria of germicidal efficiency, it is essential to have available a readily reproducible bacterial suspension of relatively known concentration and reasonably uniform resistance.

Although vegetative cells of young cultures of *Salmonella typhi* and *Staphylococcus aureus* have long been employed for evaluation of germicidal efficiency, their use has been beset with many pitfalls and inconveniences to which spores are not subject. The necessity for preparing young broth cultures of definite age imposes inconveniences and limitations as to the time when experiments may be carried out. The inexplicable variations in physiological state and cultural appearance which crop up in serial broth cultures are familiar to us all and that they are reflected in variations in resistance to disinfection has been a common experience.

The use of spore suspensions for studying the mechanism of disinfection and the effects of environmental factors on germicidal efficiency has many advantages, among which may be listed the following:

(1) A wider range of concentrations of test germicides, temperatures and reactions, can be employed than with vegetative cells.

(2) Because of the very much lower rates of deaths of spores, the course of

disinfection can be observed with a high degree of reliability, whereas this is frequently impossible for vegetative cells except within a very narrow range of experimental conditions.

(3) The necessity for daily serial transfers is eliminated, when employing spores, and preservation of the test culture requires little attention.

(4) Of particular significance, however, is the fact that a test bacterial spore suspension of relatively known concentration and uniform resistance can be prepared which, if stored in the icebox, may be employed whenever desired for a period of at least a year in a manner analogous to a chemical reagent.

The following brief remarks will be restricted to a review of observations on disinfection of spores by alkalies, hypochlorites, and chloramines employing *Bacillus metiens* as the test organism.

TABLE 6.1

*Survival and resistance of spores dried or suspended in buffered water**

DRIED SPORES			SPORE SUSPENSION		
Stored, days	Millions per 0.01 g	K.T.†	Stored, days	Millions per ml	K.T.‡
1	91	43	1	20	96
51	90	42.5	6	15	90
114	65	44	10	18	99
129	97	38	30	25	100
150	99	42	240	13	102
Mean	88.4	41.9	—	18.2	97.4
Average deviation	±9.2	±1.6	—	±3.4	±3.5
a.d.%	±10.5	±3.8	—	±18.7	±3.6

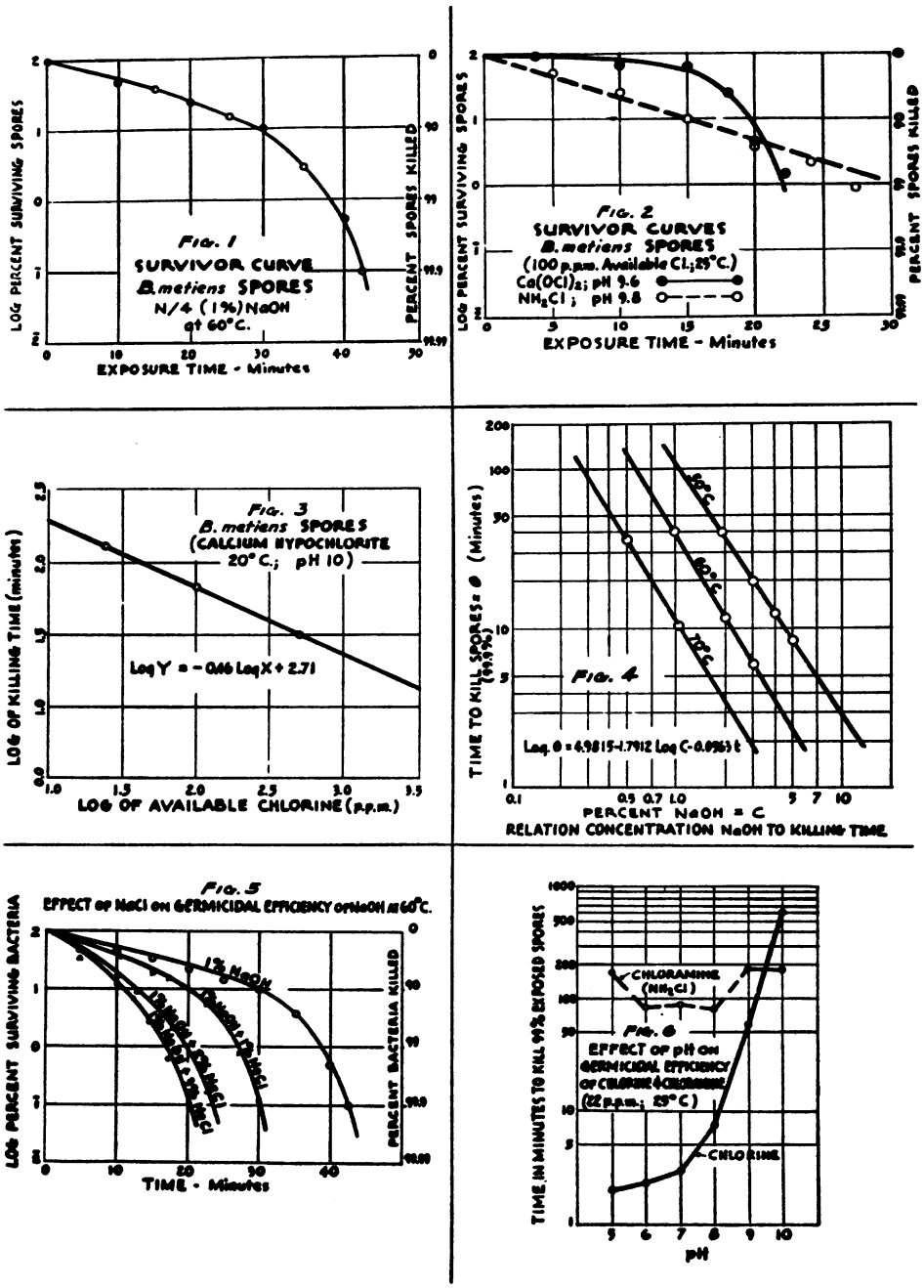
* Butterfield Formula C water.

† Time in minutes to kill 99 per cent of approximately 1,000,000 spores by N/2 NaOH at 50 C.

‡ Time in minutes to kill 99 per cent of approximately 1,000,000 spores by hypochlorite (1,000 ppm available chlorine; 20 C; pH 11.1).

METHODS

Preparation and stability of spore suspensions. Dry spores were employed in experiments with alkalies, and a suspension of spores in buffered water (Butterfield formula C) was used in observations with chlorine compounds. The dried spores were prepared in the following manner. Nutrient agar slants, inoculated from broth cultures, were incubated at 30 C for two weeks (at which time practically all cells had developed spores) after which the growth was scraped off and dried in a partial vacuum at 37 C. The mass of spores was placed in a sterile agate mortar, dry sterile lactose added, and the mixture thoroughly ground after which it was placed in a sterile weighing bottle and kept in a desiccator over calcium chloride at room temperature. The number of viable organisms per 0.01 g of dried material was determined. Desired quantities were weighed



FIGS. 6.1–6.6. Effect of NaOH, chlorine, and chloramine on destruction of spores. Details of individual tests indicated within figures.

out for use, as needed, suspended in distilled water, and passed through Whatman no. 2 filter paper to remove clumps.

In contrast to the dried spores, spore suspensions employed were prepared by washing off the growth from agar slants (20 day cultures at 30 C) in buffered water (Butterfield formula C), filtering through Whatman no. 2 paper to remove clumps, and heating the filtrate at 80 C for 10 minutes to destroy any existing vegetative cells. Plate counts were made, the suspension diluted with buffered water to approximately 20 million spores per ml and stored in the refrigerator at 6–10 C. Desired quantities were taken for disinfection tests as needed.

As may be seen from table 6.1, the number, and particularly resistance, of the spores, either dried or suspended in buffered water, remained quite constant for a considerable period of time. Variations in counts were within the range of 10 to 20 per cent and killing times varied less than 5 per cent. The stability of the dried spores and spore suspensions, with respect to resistance, makes them especially suitable for disinfection studies comparable to the use of a standardized chemical reagent.

Disinfection curves. Plots of the logarithms of the numbers of survivors against exposure times indicate that for alkalies and hypochlorites there is a distinct lag followed by a curve of increasing slope, whereas for monochloramine the plot approximates a straight line function (see figures 6.1 and 6.2). Death rates, therefore, can not be effectively employed for evaluating the effects of such factors as concentration, temperature, and reaction on the germicidal efficiencies of alkalies and hypochlorites. However, the time required to effect killing of a definite proportion (say 99 per cent) of exposed cells may be employed for such purposes. At constant temperatures, plotting the logarithms of the killing times against the logarithms of concentration of alkalies or hypochlorites employed yields straight lines so that the relationship between concentration, temperature, and killing times can be expressed mathematically as indicated in figures 6.3 and 6.4.

EFFECT OF VARIOUS FACTORS ON GERMICIDAL EFFICIENCY

Concentration of germicide. In table 6.2 are shown the killing times for various concentrations of sodium hydroxide at 50, 60, and 70 C (see also figure 6.4) and for chlorine (HOCl) and chloramine (NH_2Cl) at 20 C and pH 7. In general, reducing the concentration 50 per cent resulted in increasing killing times for chlorine by 100 per cent, monochloramine by 60 per cent, and sodium hydroxide by 240 to 300 per cent.

Temperature. In table 6.3 are shown the killing times observed for 25 ppm chlorine (HOCl) and chloramine (NH_2Cl) at pH 7 and for N/2 and N/4 NaOH at various temperatures. For each drop of 10 C the killing time rose twofold for chlorine, threefold for chloramine, and fourfold for sodium hydroxide.

Reaction. The effect of reaction (pH) is particularly significant, and in fact the controlling factor, in the germicidal efficiency of hypochlorites (free chlorine, sodium hypochlorite, and calcium hypochlorite) but not for the disinfecting properties of monochloramine or alkalies. In table 6.4 are shown the killing

times for normal solutions of sodium carbonate, phosphate, and hydroxide at 70 C. The respective killing times were 118 minutes, 5 minutes, and 0.6 minutes when the corresponding reactions were pH 11.35, pH 12.15, and pH 13.43. It is evident that sodium hydroxide was by far the most efficient germicide, and at first sight it might appear that the reaction (pH) could be employed as a meas-

TABLE 6.2

Effect of concentration on germicidal efficiency

CHLORINE (20 C; pH 7.0)		CHLORAMINE (NH_2Cl) (20 C; pH 7.0)		NaOH			
				Concentration	Killing times*		
Available chlorine, ppm	K.T.*	Available chlorine, ppm	K.T.*		50 C	60 C	70 C
6.3	11.7	11.3	110	N/8			37.4
12.4	5.4	23.6	89	N/4		46.8	10.7
23.0	3.0	44.7	41	N/2	41.7	11.7	
48.6	1.4	86.4	27	N/1	12.4		
Fold increase†....	2.0		1.6		3.4	4.0	3.5

* K.T.—Time in minutes to kill 99 per cent exposed spores of *Bacillus metiens*.
† Number of fold increase in killing time if concentration is halved.

TABLE 6.3

Effect of temperature on germicidal efficiency

TEMP C	CHLORINE (25 ppm; pH 7.0)	CHLORAMINE (25 ppm; pH 7.0)	NaOH N/4	NaOH N/2
	Killing Time*			
0	12.9			
10	6.2			
20	3.0	89.0		
30	1.4	29.5		
40		8.6		
50		2.9		41.7
60			46.8	11.7
70			10.7	
Q_{10}	2.1	3.1	4.4	3.6

* Time in minutes to kill 99 per cent exposed spores of *Bacillus metiens*.

ure of germicidal efficiency of alkalies. For each of the alkalies studied, considered individually, the germicidal efficiency is a direct function of the hydroxyl-ion concentration; but different alkalies at the same reaction (pH) do not have the same sterilizing powers.

As is evident from the data of table 6.5, at pH 12.15 sodium hydroxide (NaOH) required 36 minutes compared with only 5 minutes for trisodium phos-

phate (Na_3PO_4) to effect a reduction of 99 per cent of exposed spores; at pH 11.35 it required 220 minutes for sodium hydroxide as compared with only 118 minutes for sodium carbonate (Na_2CO_3). Thus, whereas at a stipulated normality, sodium hydroxide was the most efficient and sodium carbonate the least efficient, the reverse was true when the alkalies are compared at the same reaction (pH).

TABLE 6.4

Comparative germidical efficiencies of normal solutions of different alkalies

ALKALI	CONCENTRATION (PER CENT AS NaOH)	pH	K.T.*
Na_2CO_3	4	11.35	118
Na_3PO_4	4	12.15	5
NaOH	4	13.43	0.6

* Time in minutes to kill 99 per cent exposed spores of *Bacillus metiens* at 70 C.

TABLE 6.5

Germicidal efficiencies of different alkalies at the same reaction (pH)

pH	11.35	12.15	13.0
ALKALI	KILLING TIME IN MINUTES*		
Na_2CO_3	118	—	—
Na_3PO_4	163	5	—
NaOH	220	36	12
Commercial washing powder	—	—	6.3

* To kill 99 per cent exposed spores of *Bacillus metiens* at 70 C.

TABLE 6.6

Effect of added sodium salts on germicidal efficiency of NaOH

PER CENT ADDED SALT†	KILLING TIMES* OF N/4 NaOH WITH		
	NaCl	Na_2CO_3	$Na_3PO_4 \cdot 12H_2O$
None	42.5	42.5	42.5
1	30.6	29	34.9
2	23.4	21.9	28
3	19.9	20	24.7

* Time in minutes to kill 99 per cent exposed spores of *Bacillus metiens* at 60 C.
† The salts alone (up to 3%) had no appreciable germicidal effects.

Table 6.6 and figure 6.5 show the effects of additions of salts with a common ion (NaCl, Na_2CO_3, $Na_3PO_4 \cdot 12H_2O$) on the germicidal properties of N/4 (NaOH) at 60 C. Addition of these sodium salts did not measurably affect the reaction (pH), but the killing times were appreciably reduced. Thus, the addition of 3 per cent sodium chloride (which by itself had no affect on the spores) to 1 per cent sodium hydroxide reduced the killing time from 42.5 minutes to 19.9 min-

utes or by about 55 per cent. It is evident, therefore, that the germicidal efficiency of alkalies is influenced to a marked degree by factors other than the hydroxyl-ion concentration. It is suggested that the concentration of undissociated sodium hydroxide which penetrates the cells, rather than the hydroxyl-ion concentration, may be the controlling factor in disinfection with alkalies.

In contrast to what was observed with alkalies, the reaction is definitely the determining factor in the germicidal properties of free chlorine. In table 6.7 and figure 6.6 are shown the killing times of chlorine and chloramines (25 ppm available chlorine at 20 C) at various reactions (pH 5 to pH 10). It will be noted that the efficiency of monochloramine (NH_2Cl) is only slightly affected by

TABLE 6.7

Effect of reaction on germicidal efficiency of chlorine and chloramine (NH_2Cl)

	KILLING TIME*					
	pH					
	5	6	7	8	9	10
Chlorine	2.1	2.3	3.0	7.6	58	570
NH_2Cl	168	85	89	83	182	186

* Time in minutes to kill 99 per cent spores of *Bacillus metiens* exposed to approximately 25 ppm available chlorine at 20 C.

TABLE 6.8

Germicidal efficiency of calcium hypochlorite

CONCENTRATION OF AVAILABLE CHLORINE (PPM)	REACTION (pH)	K.T.*
1,000	11.3	64
1,000	7.3	<0.33
100	10.4	70
20	8.2	5

* Time in minutes to kill 99 per cent spores of *Bacillus metiens* at 25 C.

changes in reaction—killing times of 83 to 85 minutes in the range pH 6 to pH 8 rose to 168 minutes at pH 5 and to 182 to 186 minutes at pH 9 and 10, respectively. Although for monochloramine there seems to be an optimum reaction in the range of neutrality with decreased efficiency as the acidity or alkalinity increases, the killing time with chlorine rose slowly and progressively from 2.1 minutes at pH 5 to 7.6 minutes at pH 8, then rose rapidly to 58 minutes at pH 9 and 570 minutes at pH 10.

Alkaline hypochlorites as obtained on the market are stabilized with calcium or sodium hydroxide, or with sodium carbonate, and frequently have a reaction between pH 11 and pH 12. In table 6.8 it will be noted that by merely adjusting the reaction the killing time for 1,000 ppm $Ca(OCl)_2$ dropped from 64 minutes at pH 11 to less than 20 seconds at pH 7.3.

SUMMARY AND CONCLUSIONS

The advantages of spores, as compared with vegetative cells, as test reagents for studies on factors affecting efficiency of germicides have been presented. The fact that dried spores of *Bacillus metiens*, or suspensions of spores in buffered water, could be stored for periods of over a year without appreciable change in their resistance to alkalies or chlorine compounds is considered to be of particular significance, because it becomes possible to utilize such suspensions in a manner analogous to a chemical reagent whenever desired for disinfection studies.

Plots of the logarithms of the numbers of survivors against periods of exposure to monochloramine yielded straight lines indicating constant rates of death, whereas, when exposed to alkalies or hypochlorites, there was observed a distinct, and frequently very prolonged, lag followed by increasing slopes or death rates.

Mathematical expressions for the relations between temperature, concentration, and killing times are presented.

The effect of reaction (pH) is the controlling factor in the germicidal efficiency of hypochlorites (free chlorine, sodium hypochlorites, calcium hypochlorites) but not for the disinfecting properties of monochloramine or alkalies. The germicidal efficiency of free chlorine or hypochlorites decreases very rapidly with increasing alkalinity. With monochloramine, reactions in the range of pH 6 to pH 8 are optimum, the killing times increasing for both more acid and more alkaline solutions.

The germicidal efficiency of alkalies is markedly influenced by factors other than hydroxyl-ion concentration. Therefore, it is suggested that the concentration of undissociated sodium hydroxide which penetrates the cell, rather than the hydroxyl-ion concentration, may be the controlling factor in disinfection with alkalies.

SELECTED BIBLIOGRAPHY

ARNOLD, C. R. AND LEVINE, M. 1942 Evaluation of germicidal properties of sodium hydroxide and alkaline washing compounds. Iowa State College J. Sci., **4**, 519–538.

BUTTERFIELD, C. T. 1932 The selection of a dilution water for bacteriological examinations. J. Bact., **23**, 355–368.

CHARLTON, D. AND LEVINE, M. 1937 Germicidal properties of chlorine compounds. Iowa Eng. Exptl. Sta., Bull. **132**.

FOSTER, J. W. AND WOODRUFF, H. B. 1944 Microbiological aspects of penicillin. VI. Procedure for the cup assay for penicillin. J. Bact., **47**, 43–58.

LEVINE, M., PETERSON, E. E., AND BUCHANAN, J. H. 1927 Germicidal efficiency of sodium hydroxide, sodium carbonate and trisodium phosphate at the same H-ion concentration. Ind. and Eng. Chem., **19**, 1338–1340.

LEVINE, M., TOULOUSE, J. H., AND BUCHANAN, J. H. 1928 Effect of addition of salts on the germicidal efficiency of sodium hydroxide. Ind. and Eng. Chem., **20**, 179–183.

LEVINE, M., BUCHANAN, J. H., AND LEASE, G. 1927 Effect of concentration and temperature on germicidal efficiency of sodium hydroxide. Iowa State College J. Sci., **1**, 379–394.

RUDOLPH, A. S. AND LEVINE, M. 1941 Factors affecting the germicidal efficiency of hypochlorite solutions. Iowa Eng. Exptl. Sta., Bull. **150**.

WEBER, G. R., BENDER, R., AND LEVINE, M. 1940 The effect of ammonia on the germicidal efficiency of chlorine in neutral solutions. J. Am. Water Works Assn., **32**, 1904–1912.

WEBER, G. R. AND LEVINE, M. 1944 Factors affecting germicidal efficiency of chlorine and chloramine. Am. J. Pub. Health, **34**, 719–728.

PART VII. EFFECT OF FATTY ACIDS ON THE HEAT RESISTANCE OF CLOSTRIDIUM BOTULINUM SPORES

(Abstract)

H. SUGIYAMA

The heat resistance of *Clostridium botulinum* spores grown in a broth medium supplemented with fatty acids has been studied. One of the higher concentrations of each of the fatty acids which allowed the formation of sufficient numbers of spores to perform tests on heat resistance was used, the concentrations ranging from 0.01 M for the shorter chained acids to 0.0005 M for the higher homologues. The details of the experimental method and the actual data are to be found elsewhere (92).

The addition of acetate, propionate, or butyrate to the sporulating medium resulted in spores of slightly greater thermostability than those obtained from the control medium, the heat resistances increasing with the chain length of the fatty acids. Variable results were obtained with caprylate, although in the majority of the experiments an increased thermal tolerance of the spores was observed. The thermal tolerance of spores developing in broth containing valerate was markedly enhanced. Laurate, however, was toxic so that growth and sporulation of *C. botulinum* were negligible.

Palmitate, stearate, and oleate gave striking increases in the heat resistance of the spores. In general, where enhanced resistance of the spores resulted, the longer the fatty acid chain, the greater was the increase in heat tolerance. With the C18 fatty acids, however, an increase in the number of double bonds beyond that in oleic acid caused a progressive decrease in resistance.

The significance of the fatty acids in the development of heat resistance of the spores is emphasized by the fact that spores produced in the basal broth medium which had been extracted with chloroform or petroleum ether were more susceptible to heat than those obtained from the untreated medium. The addition of undenatured serum albumin to the casitone broth resulted in spores of diminished thermal tolerance; moreover, the formation of spores of enhanced heat stability which should occur in medium fortified with oleate was antagonized by the addition of the serum albumin.

The fatty acids appear to be incorporated in the spores in some way during their formation since spores formed in the presence of palmitate have greatly increased heat resistance although the addition of palmitate to the matured spores is without effect. Spores from the control medium washed with chloroform showed no significant change in the thermal tolerance, but the excess fatty acid in the spore suspension obtained from medium supplemented with the fatty

acid had to be washed with chloroform to remove the inhibitory effect of the fatty acid on spore germination. Since 0.01 M acetate did not increase the thermostability to the same degree as 0.001 M palmitate, a breakdown of the fatty acids to acetate did not seem to occur.

The mechanism by which the fatty acids exert their influence on the heat resistance of the spores can only be conjectured. Certain observations can be adduced, however, which may possibly bear on this effect, in particular from the point of view that the death of the heated spores is due to the denaturation of proteins. Fatty acids protect certain proteins like serum albumin from heat denaturation, the longer fatty acids, within the series tested, being more effective than equal quantities of shorter chained fatty acids. Thus, the possibility that the fatty acids exert their effect in spore heat resistance by a similar mechanism is an interesting possibility.

Other possibilities should not be ignored. The localized absence of moisture is probably the basis for the greater heat tolerance of organisms heated in fats as compared to aqueous solutions. If sporulation in media containing fatty acids results in a spore wall or membrane of greater lipoidal content and, therefore, less permeable to water, then an increase in the heat resistance may result. The possibility that the fatty acids may act by their surface active property should also be recognized.

Part VIII. Evaluation of Heat Resistance Data for Bacterial Spores

HOWARD REYNOLDS and HAROLD LICHTENSTEIN

Those who have had occasion to undertake heat resistance studies with bacterial spores are aware that these determinations are not highly precise. Replicated determinations, made under conditions that are as nearly identical as possible, often provide data exhibiting considerable variation. It is not necessary to make exhaustive search of the literature to find examples of differences in thermal death times or thermal death rates that have been interpreted as demonstration of real differences in heat resistance of spore suspensions, and other papers where differences of comparable magnitude are reported from replicated determinations on a single spore preparation. Different laboratories working with the same organism not infrequently arrive at diverse values for the constants defining its thermal resistance. It becomes important, therefore, to consider how such differences can be evaluated, the extent to which significance may be attached to observed variations in thermal resistance data, and the effect of such evaluation upon the interpretation of experimental data.

It is proposed, therefore, to present some examples of data, obtained with spore suspensions of Cameron's putrefactive anaerobe (P.A. 3679), to illustrate a possible approach to the problem of their evaluation. Definitions of certain technical terms to be used in the discussion may be useful. The D value of a thermal death rate curve is defined as the time in minutes required for the curve to pass through one logarithmic cycle when the logarithms of numbers of survivors are plotted against corresponding times. The z value of a thermal death time curve also describes slope and is defined as the number of degrees traversed

by the curve in passing through one log cycle when either D values or thermal death times are plotted on the log scale of semilogarithmic paper against corresponding temperature on the linear scale. Thus, a thermal death time curve is completely defined by its z value and a point representing the thermal death time at a given temperature. Since D values must be estimated on the basis of some arbitrarily selected degree of destruction, curves plotted through D values have slope but are without fixed position. They have, therefore, been called phantom thermal death time curves (3).

EXPERIMENTAL METHODS

Spore suspensions of P.A. 3679 were prepared by culturing the organism in 10 per cent egg-meat medium (Difco) at 30 C for 3 weeks, filtering the medium through glass wool, centrifuging the filtrate, and suspending the spores in a small volume of sterile 0.5 per cent peptone solution. Concentrated suspensions, containing approximately 3×10^8 spores per ml, were distributed in 2 to 4 ml quantities in TDT (thermal death time) tubes which were sealed and heated for 20 minutes at 100 C (84) and held under refrigeration until used. Dilutions of the concentrated suspensions were prepared for thermal death rate and thermal death time determinations by shaking mechanically with sterile sand.

Thermal death time determinations were made by heating 2 ml aliquots of suspensions sealed in pyrex TDT tubes following the method of Bigelow and Esty (8). Heated suspensions were subcultured in tryptone yeast extract agar using flat oval culture tubes (73). The inoculated agar was solidified quickly by immersing tubes in ice water and then was layered with approximately one inch of sterile 1.5 per cent agar. Recovery tubes were incubated at 30 C for a minimum period of 3 weeks. Spore concentration was 100,000 per ml. Heating was carried out in an oil bath equipped with a stirrer and thermostatically controlled to 0.3 C, and heating times were corrected for the experimentally determined heating lag (83).

Thermal death rate determinations were made in essentially the same manner as the death time determinations except that tubes were removed at shorter intervals from the oil bath, and viable counts in tryptone yeast extract agar were made on appropriate dilutions of the heated suspensions. Counts were replicated a minimum of four times, and chi square values for the resulting data were determined. Data were not used when excessively high chi square values indicated that means of replicated counts were unreliable (35).

Recovery medium used in the foregoing rate and time determinations had the following composition: 1.5 per cent tryptone (Difco), 0.5 per cent yeast extract (Difco), 0.1 per cent sodium thioglycollate (BBL), 1.5 per cent agar (Difco), pH adjusted to 7.4.

VARIATION IN THERMAL DEATH RATE DATA

In numerous reports on thermal death rate studies, inferences have been drawn from the results of single determinations. Such inferences are questionable when determinations are not adequately replicated. In figure 8.1 the results

of seven death rate determinations, carried out independently at 115 C in neutral M/15 phosphate buffer, are summarized. Mean log percentages of survivors, based on interval survivor counts, are plotted against heating time in minutes. This curve is typical of the type of results we have obtained with spore suspensions of P.A. 3679. The death rate increased during the first intervals of heating, the relationship between log per cent survivors and heating time approaching linearity after approximately 60 per cent destruction of the spores initially present. With such data it is appropriate to apply the methods of regression analysis to determine which portions are consistent with an assumed linear relationship, and to evaluate variations in the approximately linear data.

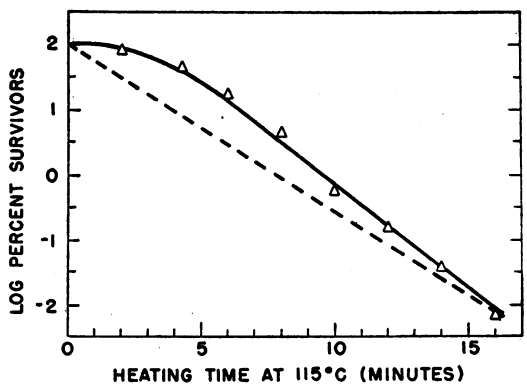


FIG. 8.1. Log per cent survivors of P.A. 3679 spores heated at 115 C in M/15 PO_4 buffer, pH, 7.0. Average of seven determinations.

In figure 8.1 the curve for the first 4 minutes is represented by a freehand line. Over the range of 4 to 16 minutes a least squares deviation line has been fitted by regression methods using the following formula:

$Y_e = \bar{Y} + b_{yx}(X - \bar{X})$ where

Y_e is the estimated value of log per cent survivors at any given time X

$\bar{Y}$ is the mean log per cent survivors = 0.178

b_{yx} is the regression coefficient = −0.324 (average decrease per min in log per cent survivors)

$\bar{X}$ is mean heating time = 9.05 min.

A summary of the variance analysis for regression of log per cent survivors of P.A. 3679 spores on heating time at 115 C is presented in table 8.1. Since over the range of 2 to 16 minutes the variance ratio of mean square for deviations from linear regression to mean square for error of 2.37 is significant, we are led to the conclusion that these data for log survivor counts and heating times are not linearly related. From 4 to 16 minutes, however, the mean square for deviations from linear regression is smaller than the error term. Over this latter range, therefore, the data are consistent with an assumption that the relationship is linear; i.e., they are insufficient in precision or quantity, or both, to

demonstrate that observed deviations from the calculated regression line are real. If similar analyses are made of data from single determinations, significant variances are sometimes observed so that no part of the curve appears to be linear; but these variances may become nonsignificant when the data from repeated determinations are analyzed. Such findings are attributable to the fact that with single rate determinations, observed error variance is associated only with differences among replicate survivor counts, the error term is smaller than when it is a resultant of all experimental variables, and an overly precise estimate is obtained of the accuracy with which any given point on a death rate curve can be established.

TABLE 8.1

Analysis of variance of regression of log per cent survivors of P.A. 3679 spores on heating time

SOURCE OF VARIATION	2–16 MINUTES			4–16 MINUTES		
	Sum of squares	Degrees of freedom	Mean square	Sum of squares	Degrees of freedom	Mean squares
Total	76.908	44		58.461	37	
Between classes	73.109	7		54.736	6	
Linear regression	71.648	1		54.298	1	
Class deviations from linear regression	1.461	6	0.2435*	0.438	5	0.0876
Within classes (error)	3.799	37	0.1027	3.725	31	0.1202

M/15 PO_4 buffer, pH 7.0; temperature, 115 C.

* Significant mean square at 5 per cent level.

The foregoing calculated regression coefficient ($b_{yx} = -0.324$) is the decrease per minute in log per cent survivors; the D value for the linear portion of the curve (4 to 16 minutes) is its reciprocal, 3.09. The dotted line in figure 8.1, representing the curve that would obtain if the entire survivor curve were linear, has a larger D value of 3.93.

Returning again to the regression coefficient of -0.324, it was, as noted, calculated from data for which means only are presented. Because individually determined values fluctuated around these means, some degree of uncertainty is associated with each mean and, therefore, with the regression coefficient calculated from the several determinations at each time interval. The appropriate calculations provide 0.0149 as the estimate of the standard error of this regression coefficient. From this standard error we can set up confidence limits for the regression coefficient and correspondingly for the derived D values. Estimated in this manner from the survivor data at 115 C, the 1 per cent confidence limits of the D value for spore suspensions of this organism under the described conditions are 2.74 to 3.53. On the basis of these data it may be stated with about 99 per cent confidence that the true D value lies somewhere in this interval. While it cannot be concluded that the true value is defined more accurately than by the estimated limits, the mean of 3.09 is the best estimate of this value

that is provided by the data. Assuming, therefore, that the true D value is more closely approximated by the observed mean of 3.09 than by its one per cent confidence limits (2.74 and 3.53), it would be reasonable to expect that additional determinations would yield D values tending to average around 3.09, but too much significance could not be attached to mean values as low as 2.74 or as high as 3.53. In other words, D values at 115 C differing by as much as 0.79 would not, with data of the precision obtained here, be substantial proof of a real difference in death rates if significance of differences is evaluated on a probability level of $p = 0.01$. At the 5 per cent probability level, differences required for significance would be correspondingly smaller and confidence limits narrower.

TABLE 8.2

Thermal death rate data of P.A. 3679 spores

TEMPERATURE	NUMBER OF DETERMINATIONS	MEAN D VALUE*	b_{yx}	D FROM REGRESSION†	CONFIDENCE LIMITS OF D FROM REGRESSION†	
					1 per cent level	5 per cent level
C		*minutes*		*minutes*	*minutes*	*minutes*
107	4	19.88	−0.068	14.71	13.04–16.86	13.44–16.23
111	4	9.08	−0.140	7.13	6.76– 7.54	6.85– 7.42
115	7	3.93	−0.324	3.09	2.74– 3.53	2.82– 3.41

Spores heated in M/15 PO_4 buffer, pH 7.0.

* $D = \dfrac{\text{time}}{\text{log initial number} - \text{log final number}}$.

† $D = \dfrac{1}{b_{yx}}$.

Table 8.2 presents data from death rate determinations at 107, 111, and 115 C. D values calculated on the assumption of completely exponential survivor curves are shown in the third column. In the fourth column are regression coefficients for those portions of the curves over which deviations from linearity were not significant. D values calculated from these regression coefficients and their estimated confidence limits are shown in the last three columns.

THERMAL DEATH TIME CURVES FROM DEATH RATE DATA

The two sets of D values in table 8.2 provide estimates of the phantom thermal death time curve for P.A. 3679 spores in neutral phosphate buffer. When the least squares deviation lines were fitted to the data, the D values calculated from regression coefficients yielded a curve with a z value of 11.8 C which is not significantly different than the z of 11.4 C for the curve, based on the D values calculated on the assumption that the survivor curves were exponential over their entire ranges. Since position of phantom thermal death time curves is without significance (3), it is evident that D values, based on the latter portions of survivor curves that exhibit an initial lag, can yield the same thermal death time curve as D values estimated from the same data on the assumption that the entire survivor curve is linear.

When several estimates of D values are available at each of three or more lethal temperatures, a logical approach to the problem of estimating variance in the slope of thermal death time curves is offered. In table 8.3, the means of D values determined at 3 different temperatures are presented together with their standard deviations. The least squares deviation line fitted to the individual values, for which means only are shown, has a z value of 11.9 C and is the best estimate provided by these data of the slope of the thermal death time curve for P.A. 3679 spores in neutral M/15 phosphate buffer. From the standard error of the slope of the fitted line, the estimated one and five per cent confidence limits of this z value are 10.5 and 13.8 C and 10.9 to 13.2 C, respectively. With variance of the observed magnitude, therefore, and significance based on a probability of $p = 0.01$, z values as low as 10.5 C or as high as 13.8 C would not be considered as significantly different from each other or from the mean of observed values when based on single or limited determinations. It may also

TABLE 8.3

Mean D values from linear portions of thermal death rate curves for P.A. 3679 spore suspensions

TEMPERATURE	NUMBER OF DETERMINATIONS	D VALUE*	
		Mean	Standard deviation
C		*minutes*	*minutes*
107	4	14.75	1.35
111	4	7.44	0.60
115	7	3.20	0.43

Spores heated in M/15 PO_4 buffer, pH 7.0.

* $\frac{1}{b_{yz}}$.

be noted that this z value is, for practical purposes, identical to that derived from the regression coefficients calculated from the combined death rate data.

In the foregoing discussion, data have been presented which show that, when P.A. 3679 spore suspensions were heated in neutral phosphate buffer, the relationship between logarithms of numbers of surviving spores and heating times became approximately linear only after destruction of half or more of the spores initially present. It has been emphasized, however, that these findings do not constitute proof that even the latter portions of survivor curves were exponential, but only that such an assumption is consistent with the data presented. As will be pointed out in the following discussion, more precise or more extensive data may lead to a different interpretation.

D VALUES FROM THERMAL DEATH TIME DATA

In carrying out thermal death time determinations in our laboratory it has been the practice to subculture heated suspensions in agar medium in flat oval culture tubes. On the assumption that survivor curves are linear, this technique provides for the estimation of D values from initial spore counts and counts on

subcultures of one or more of those portions of suspensions which have been heated for periods of time that are somewhat shorter than required to give complete sterility.

In examining data collected in this way over a period of several years, it has been observed that D values, calculated from initial counts on P.A. 3679 spore suspensions and from survivor counts made after heating for two or more periods of time just short of those giving complete sterility (less than one survivor per 2 ml of suspension), exhibit a general tendency to increase with time of heating. Typical examples of such series of D values are presented in table 8.4.

To permit more precise evaluation of this question, data from 52 individual thermal death time determinations at 115 C providing a total of 165 values of D were examined. Because these D values were derived in some instances from

TABLE 8.4

*D values for P.A. 3679 spores**

HEATING TIME	DETERMINATION NUMBER									
	74	79	80	80a	81	83	103	112	112a	158
minutes	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*
12		3.53	3.06	3.40		3.40	3.25	3.30	3.56	
16		3.71	3.74	3.52	3.79	3.62	3.59	3.45	3.52	3.64
18	4.01									4.08
20	4.40	4.19	4.19	4.15	4.55	4.47	3.77	3.65	3.46	4.05
22	4.46									
24					4.64			4.15	4.15	4.60

Spores heated at 115 C in M/15 PO_4 buffer, pH 7.0.

* $D = \frac{\text{time}}{\text{log initial number} - \text{log final number}}$. Calculated from counts at intervals just short of those giving complete sterility (less than 1 survivor per 2 ml of suspension).

suspensions of differing resistance, and in others from determinations in which degree of resistance was modified by experimental conditions, it was necessary to obtain an estimate of the *within determination* heating time—D value relationship that was unbiased by over-all variations in resistance. To this end, D values and their associated heating times were subjected to covariance analysis using methods outlined by Snedecor (90). The results showed that, independently of their average value, there was a highly significant and positive correlation between observed D values and their corresponding heating times. The probability of chance occurrence of the calculated correlation coefficient of +0.64 with 112 degrees of freedom is considerably less than 0.01. The regression coefficient provided 0.053 as an estimate of the average increase in D value per minute of heating time in the neighborhood of complete spore destruction.

Similar evidence of decreasing death rates was provided by laboratory data used in preparing an earlier report (83). These data provided two or more D

values for P.A. 3679 spores dispersed in each of eight different low-acid vegetables and processed at 250 F in thermal death time cans. Once again, since the magnitude of observed D values varied with the product in which the spores were heated, the data were analyzed by the method of covariance. These data likewise showed that, independently of average values, there was a highly significant and positive *within determination* correlation ($r = 0.93$, $df = 15$) between D value and heating time. The average increase in D value per minute of heating time was estimated as 0.18.

Additional evidence of increasing D values with heating time is provided by data appearing in two recent publications (27, 91). Values reported by Stumbo, Murphy, and Cochran (91) for P.A. 3679 spores in a puree of peas exhibit a similar tendency to increase with the time of heating. To provide a sufficient number of values for analyses that are representative of a given temperature, the following equation, based on a thermal death time curve with the reported z value of 17.3 F, was used to convert D values and heating times at 220, 250, 260, and 270 F to their equivalent 240 F value:

$$t_{240} = \log^{-1}\left[\frac{T_x - 240}{17.3} + \log t_x\right]$$

where: t_{240} = ordinate, expressed in minutes, of the 240 F point on the thermal death time curve.

t_x = ordinate, expressed in minutes, of the point on the thermal death time curve at another temperature, T_x.

Analysis of the resulting data yielded a highly significant and positive correlation ($r = +0.81$, $df = 11$) between D value and heating time. The regression coefficient provided 0.12 as an estimate of the average increase in D value per minute of heating time.

Data reported by Desrosier and Esselen (27) were analyzed in a similar manner. Covariance analyses of these time—D value data, after conversion to their 240 F equivalents, yielded a highly significant positive correlation coefficient of 0.89 (17 degrees of freedom) between D value and heating time. The average increase in D value per minute as estimated by the regression coefficient is 0.12.

DISCUSSION

Thermal death time and thermal death rate data reported here for P.A. 3679 spores suspended in neutral phosphate buffer have been shown to be inconsistent with the assumption that survivor curves are exponential. The results indicate an initial phase of accelerating rate of death during which half or more of the spores initially present become nonviable; a second phase during which the data are compatible, within the limits of observational error, with an assumed exponential relationship between heating time and spore destruction; and a final phase during which increasing D values indicate a decreasing rate of death as the last few surviving spores are inactivated.

While the apparently obvious may not be the correct interpretation, other explanations of the reported data require assumptions that are not readily amenable to experimental verification. No evidence of significant clumping of spores in the P.A. 3679 suspensions used in these studies has been found either by microscopic examination or by other tests. Apparent spore counts have remained unchanged during mechanical shaking of spore suspensions with sand for periods up to one hour and during exposure to high energy ultrasonic vibrations at frequencies of 400 and 1,500 kilocycles for periods up to 20 minutes. Since these treatments could be expected to result in partial or complete dispersal of spore clumps and corresponding increases in apparent spore counts, the results are evidence of the absence of significant numbers of clumps in the suspensions used.

In addition to clumping of spores (81, 82) an apparent initially accelerating rate of death could be expected if unactivated suspensions of heat activatable spores were used. The data reported here, however, were all obtained with suspensions preheated at 100 C for 20 minutes, a heat treatment shown by other studies (84), to provide for maximum viable counts on spore suspensions of the organism used.

Survivor curves exhibiting an initial phase of accelerating rate of death pose no serious problems in the interpretation, by currently used methods for process evaluation, of thermal death time data in terms of lethalities of heat treatments. If, following such an initial phase, the remaining portions of survivor curves become exponential, an estimate of the sterilizing value of a given heat treatment would err only with respect to the initial number of organisms that the process would be expected to destroy. Thermal death rate curves characterized by declining rates of death during destruction of the organisms that survive the longest present more serious difficulties. Demonstration that such curves are typical would invalidate inferences, based on extrapolation of thermal death rate curves, concerning reductions in numbers of survivors to values below those that are determinable by available bacteriological techniques. Data presented here, derived from determinations carried out in the Bureau's laboratory as well as from other sources, indicate that there does occur an observable decrease in the rate of death as the end point of complete destruction of a population of spores is approached. This apparent decrease in death rates is evidenced by the regular occurrence of positive and statistically significant correlations between estimated D values and heating times.

The results are evidence, therefore, that the order of death of P.A. 3679 spores when exposed to heat is not purely a matter of chance but is determined entirely or in part by a distribution of resistances among individuals of the spore populations in question. Withell (101) has also concluded that available data are in accord with the assumption of a distribution of resistances among individuals of bacterial or bacterial spore populations and has shown that this distribution, when measured by logarithms of survival times, approximates the normal.

SUMMARY

Thermal death time and thermal death rate data for P.A. 3679 spores have been presented, and methods for statistical evaluation of variation in such data have been proposed. The results indicate that experimentally determined estimates of thermal resistance constants may be expected to have rather wide limits within which variations are of questionable significance.

Data presented are not consistent with the assumption that thermal death rate curves for P.A. 3679 spores are exponential. Increasing rates of death during the first intervals of exposure of spores to lethal heat and the regular observance of increasing D values in the neighborhood of complete spore destruction indicate the sigmoid type of survivor curve characteristic of populations made up of individuals of varying resistance.

ACKNOWLEDGMENT

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Part IX. Relative Resistance of Bacterial Spores and Vegetative Bacteria to Disinfectants

(Abstract)

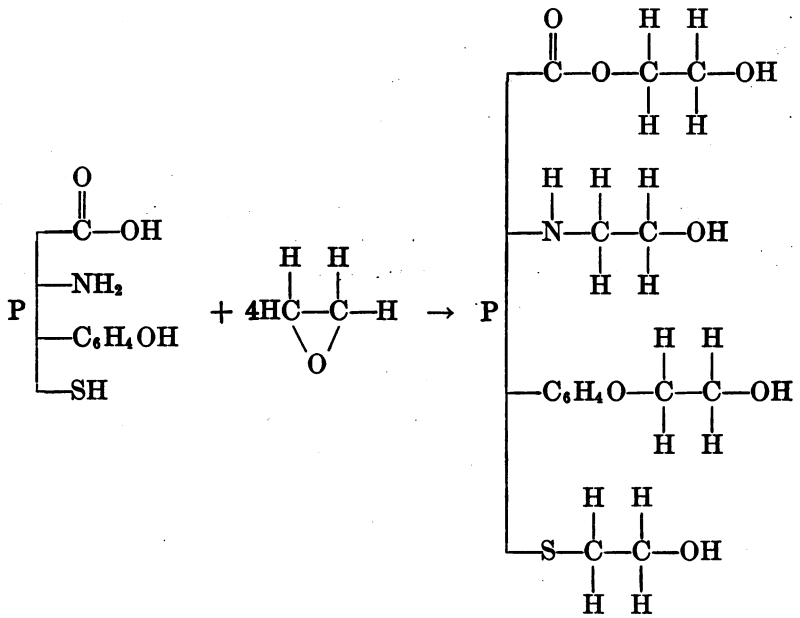
CHARLES R. PHILLIPS

This laboratory has been engaged for a number of years in a study of the effect of the vapors of various volatile organic compounds upon microorganisms. It was observed early in this work that the usual great difference in resistance towards chemical action exhibited by bacterial spores as compared with vegetative forms of bacteria was not evident with many of these vapor phase disinfectants.

In this paper an attempt is made to express this general observation in quantitative terms to determine if an insight might be gained into the mechanism by which bacterial spores protect themselves against an unfavorable chemical or physical environment.

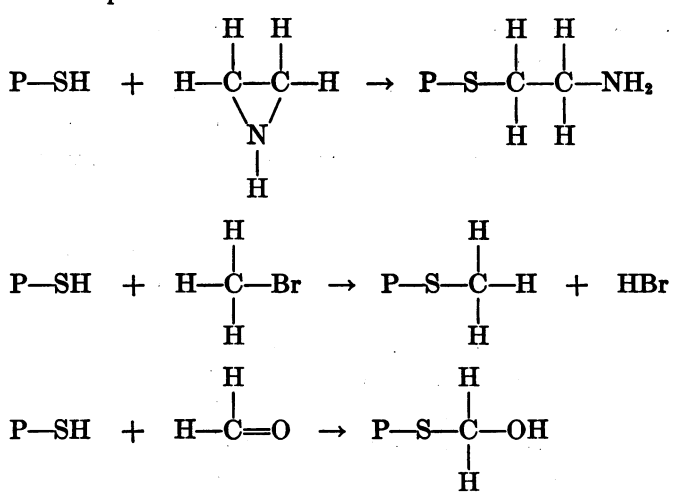
The particular vapor phase disinfectants included in this study were ethylene oxide, ethylene imine, methyl bromide, and formaldehyde, all of which in the vapor state affect dry bacteria. They also act similarly upon bacterial suspensions when in aqueous solution. The proposed mechanism of action of ethylene oxide is that of direct alkylation upon the bacterial proteins according to the

following scheme, which was shown by Fraenkel-Conrat to be true for the action of that compound upon the protein keratin.



SCHEME I

Here ethylene oxide alkylates carboxyl, amino, hydroxyl or sulfhydryl groups directly replacing a hydrogen atom with an hydroxy ethyl group. Similarly the other three compounds might alkylate to add an aminoethyl, methyl or hydroxy methyl group as shown below, using only the sulfhydryl group in proteins as an example:



SCHEME II

Data exist in this laboratory showing the effect of the first three of these compounds, both in vapor form and in solution, on a typical resistant bacterial spore, *Bacillus globigii*, and upon typical vegetative organisms *Escherichia coli* and *Micrococcus pyogenes* var. *aureus*. Similar information is available for formaldehyde, utilizing some data from the literature. Relative activity for different organisms is obtained by giving a numerical Ct value (Concentration-time) to the action of a given compound on a particular organism. This Ct value is the product of the concentration at which the disinfectant was used and the length of time required for producing a given effect, sterility in most cases. Then by comparing the Ct values for the same compound against *B. globigii* with those against *E. coli* and *M. pyogenes* var. *aureus* the ratio of the resistance of the spore former to the vegetative organisms can be obtained.

TABLE 9.1

Relative activity of certain disinfectants against bacterial spores and against vegetative cells

DISINFECTANT	RATIO OF RESISTANCE *Bacillus globigii*/*Micrococcus pyogenes* and *Escherichia coli*
Alkylating agents	
Ethylene oxide	Between 2 and 6
Ethylene imine	Between 0.5 and 10
Methyl bromide	Between 2 and 5
Formaldehyde	Between 2 and 15
Chlorine	
Sodium hypochlorite	about 10^4
Silver	
Movidyn	about 10^4
Phenol	
Trichlorophenol	about 10^3
Quaternaries	
4-Cetyl-4-methyl-morpholinium methyl sulfate	about 10^4

In table 9.1 these ratios appear for the four volatile compounds which act as alkylating agents. Note that these ratios for the relative resistance of spores *versus* vegetative cells are the same whether these compounds are acting as gasses or in liquid solution, since the spread of values includes data obtained in both these ways.

Similar comparisons were made for several typical disinfectants of various other classes. This information was calculated usually from experiments performed in this laboratory, but occasionally data from the literature were used whenever specific information was lacking here. Only orders of magnitude are reported, not precise ratios, since these vary over a considerable range when data from different experiments are used. The values all fall, however, in about the order of magnitude shown in table 9.1.

Examination of these data reveals that the alkylating disinfectants reported upon here are quite different in their action on spores than are the more commonly studied classes of disinfectants. Usually, spores are hundreds or thousands

of times more resistant to chemical disinfectants than are vegetative bacteria. In this one class, however, no very great difference appears in the resistance of bacterial spores. True, the spores are somewhat more difficult to kill, but they proved in most experiments to have only a few fold increase in resistance. This was true for all of the compounds of this type for which sufficient data are available to make the comparisons.

Some idea of the mechanism whereby spores protect themselves against chemical action may be gained from these observations. The most notable property of alkylating compounds is that they have a multiple choice of action, as exemplified in the first scheme. They can attack four or more of the reactive groups which are found in all proteins. If one such group is unexposed or blocked, alternate points of attack may not be. Probably the most vulnerable of these is the sulfhydryl group which appears in many key and essential bacterial enzyme systems. Although no general agreement has ever been reached as to the mechanism of action of various common disinfectants, the possibility of direct action, such as oxidation, insoluble salt formation, or the like, upon sulfhydryl groups has often been suggested. A folding, or reformation of the protein molecular configuration, which protects these sulfhydryl groups could well occur in spore formation, accounting for the very great difference in resistance which spores usually show over vegetative organisms. With sulfhydryl groups not in a position where they can react readily, the mechanism of killing would be greatly slowed.

With alkylating agents on spore formers, such a protection of the sulfhydryl groups would merely result in a reaction with other groups which are not so well protected but which are still necessary for metabolism. With these agents all the reactions indicated in the first scheme could be taking place simultaneously at their various reaction rates. Removal of sulfhydryl groups from the reaction might slow down the over-all attack upon the protein somewhat but not as significantly as it would if this were the only group principally involved.

REFERENCES

1. Allen, Mary B. 1950 The dynamic nature of thermophily. J. Gen. Physiol., **33**, 205–214.
2. Anderson, E. E., Esselen, W. B. Jr., and Fellers, C. R. 1949 Effect of acids, salts, sugar and other food ingredients on thermal resistance of *Bacillus thermoacidurans*. Food Research, **14**, 499–510.
3. Ball, C. O. 1943 Short time pasteurization of milk. Ind. Eng. Chem., **35**, 71–84.
4. Barron, E. S. G. and Flood, Veronica 1950 Studies on the mechanism of action of ionizing radiations. VI. The oxidation of thiols by ionizing radiations. J. Gen. Physiol., **33**, 229–241.
5. Bayne-Jones, S. and Petrilli, A. 1933 Cytological changes during the formation of the endospore in *Bacillus megatherium*. J. Bact., **25**, 261–276.
6. Behring, E. 1889 Beitraege zur Aetiologie des Milzbrandes. Z. Hyg. Infektionskrankh., **6**, 117–144.
7. Behring, E. 1889 Beitraege zur Aetiologie des Milzbrandes. VI. Ueber asporogenen Milzbrand. Z. Hyg. Infektionskrankh., **7**, 171–185.
8. Bigelow, W. D. and Esty, J. R. 1920 The thermal death point in relation to time of typical thermophilic organisms. J. Infectious Diseases, **27**, 602–617.

9. BISSET, K. A. 1950 Evolution in bacteria and the significance of the bacterial spore. Nature, **166**, 431–432.
10. BISSET, K. A. 1950 The sporulation of *Clostridium tetani*. J. Gen. Microbiol., **4**, 1–2.
11. BLAIR, E. B. 1950 Observations on bacterial spore formation in synthetic media. M.A. Thesis, University of Texas. Abstracted in Texas Repts. Biol. Med., **8**, 361.
12. BLAU, O. 1906 Ueber die Temperaturmaxima der Sporenkeimung und der Sporenbildung, sowie die supermaximalen Tötungszeiten der Sporen der Bakterien, auch derjenigen mit hohen Temperaturminima. Zentr. Bakt. Parasitenk., Abt. II, **15**, 97–143.
13. BONGERT, J. 1903–1904 Beiträge zur Biologie des Milzbrandbacillus und sein Nachweis im Kadaver der grossen Haustiere. Zentr. Bakt. Parasitenk., Abt. I, Orig., **34**, 497–507, 623–630, 772–792; **35**, 14–24, 168–201.
14. BRUNSTETTER, B. C. AND MAGOON, C. A. 1932 Studies on bacterial spores. III. A contribution to the physiology of spore production in *Bacillus mycoides*. J. Bact., **24**, 85–122.
15. BUCHNER, H. 1890 Ueber die Ursache der Sporenbildung beim Milzbrandbacillus. Zentr. Bakt. Parasitenk., Abt. I, Orig., **8**, 1–6.
16. BULLER, A. H. R. 1909 Researches on Fungi, **1**, 153–178, Longmans, Green and Co., London.
17. BURKHOLDER, P. R. AND GILES, N. H., JR. 1947 Induced biochemical mutations in *Bacillus subtilis*. Am. J. Botany, **34**, 345–348.
18. CHRISTIAN, M. I. 1931 A contribution to the bacteriology of commercial sterilised milk. Part II. The "coconut" or "carbolic" taint. A study of the causal organism and the factors governing its spore-formation. J. Dairy Research, **3**, 113–132.
19. COLLIER, R. E. AND WYNNE, E. S. 1950 Germination of spores of certain *Clostridium* species in the presence of streptomycin. Unpublished data.
20. COOK, R. P. 1932 Bacterial spores. Biol. Revs., Cambridge Phil. Soc., **7**, 1–23.
21. CURRAN, H. R., BRUNSTETTER, B. C., AND MYERS, A. T. 1943 Spectrochemical analysis of vegetative cells and spores of bacteria. J. Bact., **45**, 485–494.
22. CURRAN, H. R. AND EVANS, F. R. 1937 The importance of enrichments in the cultivation of bacterial spores previously exposed to lethal agencies. J. Bact., **34**, 179–189.
23. CURRAN, H. R. AND EVANS, F. R. 1945 Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. J. Bact., **49**, 335–346.
24. DARANYI, J. V. 1930 Das Wesen der Bakteriensporenbildung und ihre Stellung im Fortpflanzungssystem. Zentr. Bakt. Parasitenk., Abt. I, Orig., **117**, 543–547.
25. DAVIS, F. L., JR., WYSS, O., AND WILLIAMS, O. B. 1948 Studies on heat resistance. II. Comparison of resistance to heat with resistance to disinfectants. J. Bact,. **56**, 561–567.
26. DELAPORTE, BERTHE 1950 Observation on the cytology of bacteria. Advances in Genetics, **3**, 1–32.
27. DESROSIER, N. W. AND ESSELEN, W. B., JR. 1951 Attempts to increase the heat resistance of the spores of a putrefactive anaerobe by selection. J. Bact., **61**, 541–547.
28. DOAK, B. W. AND LAMANNA, C. 1948 On the antigenic structure of the bacterial spore. J. Bact., **55**, 373–380.
29. DUGGAR, B. M. AND HOLLAENDER, A. 1934 Irradiation of plant viruses and of microorganisms with monochromatic light. II. Resistance to ultraviolet radiation of a plant virus as contrasted with vegetative and spore stages of certain bacteria. J. Bact., **27**, 241–256.
30. DYRMONT, A. 1886 Einige Beobachtungen über Milzbrandbacillen. Arch. f. exper. Path. u. Pharmakol., **21**, 309–317.
31. ESTY, J. R. AND MEYER, K. F. 1922 The heat resistance of the spores of *B. botulinus* and allied anaerobes. XI. J. Infectious Diseases, **31**, 650–663.

32. EVANS, F. R. AND CURRAN, H. R. 1943 The accelerating effect of sublethal heat on spore germination of mesophilic aerobic bacteria. J. Bact., **46**, 513–523.
33. FABIAN, F. W. AND BRYAN, C. S. 1933 The influence of cations on aerobic sporogenesis in a liquid medium. J. Bact., **26**, 543–558.
34. FISCHOEDER, F. 1909 Beiträge zur Kenntnis des Milzbrandes. Zentr. Bakt. Parasitenk., Abt. I, Orig., **51**, 320–417.
35. FISHER, R. A., THORNTON, H. G., AND MACKENZIE, W. A. 1922 The accuracy of the plating method of estimating the density of bacterial populations with particular reference to the use of Thornton's agar medium with soil samples. Ann. Applied Biol., **9**, 325–359.
36. FOSTER, J. W., HARDWICK, W. A., AND GUIRARD, B. 1950 Antisporulation factors in complex organic media. I. Growth and sporulation studies on *Bacillus larvae*. J. Bact., **59**, 463–470.
37. FOSTER, J. W. AND HEILIGMAN, F. 1949 Mineral deficiencies in complex organic media as limiting factors in sporulation of aerobic bacilli. J. Bact., **57**, 613–615.
38. FOSTER, J. W. AND HEILIGMAN, F. 1949 Biochemical factors influencing sporulation in a strain of *Bacillus cereus*. J. Bact., **57**, 639–646.
39. FOSTER, J. W. AND WYNNE, E. S. 1948 Physiological studies on spore germination with special reference to *Clostridium botulinum*. IV. Inhibition of germination by unsaturated C_{18} fatty acids. J. Bact., **55**, 495–501.
40. FRIEDMAN, C. A. AND HENRY, B. S. 1938 Bound water content of vegetative and spore forms of bacteria. J. Bact., **36**, 99–105.
41. HARDWICK, W. A., GUIRARD, B., AND FOSTER, J. W. 1951 Antisporulation factors in complex organic media. II. Saturated fatty acids as antisporulation factors. J. Bact., **61**, 145–151.
42. HAYWARD, A. E. 1943 Some physiological factors in spore production. J. Bact., **45**, 200.
43. HEADLEE, M. R. 1931 Thermal death point. III. Spores of *Clostridium welchii*. J. Infectious Diseases, **48**, 468–483.
44. HENRY, B. S. AND FRIEDMAN, C. A. 1937 The water content of bacterial spores. J. Bact., **33**, 323–329.
45. HILLIER, J., KNAYSI, G., AND BAKER, R. F. 1948 New preparation techniques for the electron microscopy of bacteria. J. Bact., **56**, 569–576.
46. HILLS, G. M. 1948 The relation of optical form to the influence of amino-acids on the germination of aerobic spores. Biochem. Soc. Symposia, **1**, 86–88.
47. HILLS, G. M. 1949 Chemical factors in the germination of spore-bearing aerobes. The effect of yeast extract on the germination of *Bacillus anthracis* and its replacement by adenosine. Biochem. J., **45**, 353–362.
48. HILLS, G. M. 1949 Chemical factors in the germination of spore-bearing aerobes. The effects of amino-acids on the germination of *Bacillus anthracis*, with some observations on the relation of optical form to biological activity. Biochem. J., **45**, 363–370.
49. HILLS, G. M. 1950 Chemical factors in the germination of spore-bearing aerobes: observations on the influence of species, strain, and conditions of growth. J. Gen. Microbiol., **4**, 38–47.
50. HOLZMUELLER, K. 1909 Die Gruppe des *Bacillus mycoides* Fluegge. Ein Beitrag zur Morphologie und Physiologie der Spaltpilze. Zentr. Bakt. Parasitenk., Abt. II, **23**, 304–354.
51. HUNTER, M. E. AND DELAMATER, E. D. 1951 Nuclear phenomena in sporulation and spore germination in *Bacillus megatherium*. Bact. Proc., p. 41.
52. ITANO, A. AND NEILL, J. 1919 Influence of temperature and hydrogen ion concentration upon the spore cycle of *Bacillus subtilis*. J. Gen. Physiol., **1**, 421–428.
53. KEILIN, D. AND HARTREE, E. F. 1947 Comparative study of spores and vegetative forms of *Bacillus subtilis*. Antonie von Leeuwenhoek J. Microbiol. Serol., **12**, 115–128.

54. KNAYSI, G. 1938 Cytology of bacteria. Botan. Rev., **4**, 83–112.
55. KNAYSI, G. 1945 A study of some environmental factors which control endospore formation by a strain of *Bacillus mycoides*. J. Bact., **49**, 473–493.
56. KNAYSI, G. 1945 Investigation of the existence and nature of reserve material in the endospore of a strain of *Bacillus mycoides* by an indirect method. J. Bact., **49**, 617–622.
57. KNAYSI, G. 1946 On the process of sporulation in a strain of *Bacillus cereus*. J. Bact., **51**, 187–197.
58. KNAYSI, G. 1948 The endospore of bacteria. Bact. Revs., **12**, 19–77.
59. KNAYSI, G. 1951 Elements of Bacterial Cytology. 2nd Ed. Comstock Publishing Co., Inc., Ithaca, N. Y.
60. KNAYSI, G. AND BAKER, R. F. 1947 Demonstration, with the electron microscope, of a nucleus in *Bacillus mycoides* grown in a nitrogen-free medium. J. Bact., **53**, 539–553.
61. KNIGHT, B. C. J. G. AND FILDES, P. 1930 Oxidation-reduction studies in relation to bacterial growth. III. The positive limit of oxidation-reduction potential required for the germination of *B. tetani* spores *in vitro*. Biochem. J., **24**, 1496–1502.
62. LAMANNA, C. 1940 The taxonomy of the genus *Bacillus*. II. Differentiation of small celled species by means of spore antigens. J. Infectious Diseases, **67**, 193–204.
63. LAMANNA, C. 1942 The status of *Bacillus subtilis*, including a note on the separation of precipitinogens from bacterial spores. J. Bact., **44**, 611–617.
64. LAMANNA, C. 1942 Relation of maximum growth temperature to resistance to heat. J. Bact., **44**, 29–35.
65. LEIFSON, E. 1931 Bacterial spores. J. Bact., **21**, 331–356.
66. LWOFF, A. AND MONOD, J. 1947 Essai d'analyse du rôle de l'anhydride carbonique dans la croissance microbienne. Ann. inst. Pasteur, **73**, 323–347.
67. MAGOON, C. A. 1926 Studies upon bacterial spores. I. Thermal resistance affected by age and environment. J. Bact., **11**, 253–283.
68. MATZUCHITA, T. 1902 Zur Physiologie der Sporenbildung der Bacillen, nebst Bemerkungen zum Wachstum einiger Anaeroben. Arch. Hyg. Bakt., **43**, 267–375. Abstracted in Zentr. Bakt. Parasitenk., Abt. I, Ref., **32**, 137–139.
69. MEFFERD, R. B., JR. AND WYSS, O. 1951 The mutability of *Bacillus anthracis* spores during germination. J. Bact., **61**, 357–364.
70. MEHL, D. A. AND WYNNE, E. S. 1951 A determination of the temperature characteristic of spore germination in a putrefactive anaerobe. J. Bact., **61**, 121–126.
71. MELLON, R. R. 1926 Studies in microbic heredity. X. The agglutinin-absorption reaction as related to the newer biology of bacteria, with special reference to the nature of spore formation. J. Immunol., **12**, 355–375.
72. MIGULA, W. 1897 System der Bakterien, 1 Allgemeiner Teil. Gustav Fischer, Jena.
73. MILLER, N. J., GARRET, O. W. AND PRICKETT, P. S. 1939 Anaerobic technique—a modified deep agar shake. Food Research, **4**, 447–451.
74. MURRAY, T. J. 1931 Thermal death point. II. Spores of *Bacillus anthracis*. J. Infectious Diseases, **48**, 457–467.
75. MURRAY, T. J. AND HEADLEE, M. R. 1931 Thermal death point. I. Spores of *Clostridium tetani*. J. Infectious Diseases, **48**, 436–456.
76. MURRELL, W. G., OLSEN, A. M., AND SCOTT, W. J. 1950 The enumeration of heated bacterial spores. II. Experiments with *Bacillus* species. Australian J. Sci. Research, **3**, 234–244.
77. OLSEN, A. M. AND SCOTT, W. J. 1946 Influence of starch in media used for the detection of heated bacterial spores. Nature, **157**, 337.
78. OLSEN, A. M. AND SCOTT, W. J. 1950 The enumeration of heated bacterial spores. I. Experiments with *Clostridium botulinum* and other species of *Clostridium*. Australian J. Sci. Research, **3**, 219–233.

79. PASTEUR, L., CHAMBERLAND, C., AND ROUX, E. 1881 De l'attenuation des virus et de leur retour à la virulence. Compt. rend. acad. sci., **92**, 429–435.
80. PHISALIX, C. 1892 État asporogène héréditaire du *Bacillus anthracis*. Le Bull. méd., No. 35, 293. Cited by Migula.
81. RAHN, O. 1930 The non-logarithmic order of death of some bacteria. J. Gen. Physiol., **13**, 395–407.
82. RAHN, O. 1945 Injury and Death of Bacteria by Chemical Agents. Biodynamica Monograph No. 3, Biodynamica, Normandy, Missouri.
83. REYNOLDS, H., KAPLAN, A. M., SPENCER, F. B., AND LICHTENSTEIN, H. 1952 Thermal destruction of Cameron's putrefactive anaerobe 3679 in food substrates. Food Research, **17**, 153–167.
84. REYNOLDS, H. AND LICHTENSTEIN, H. 1949 Germination of anaerobic spores induced by sublethal heating. (Abs.) Soc. Am. Bact. Abst. of Papers, 49th Gen. Meeting, p. 9.
85. REYNOLDS, H. AND LICHTENSTEIN, H. 1950 Effect of certain growth factors on the heat resistance of anaerobic spores. Bact. Proc., p. 28.
86. ROBERTS, J. L. AND BALDWIN, I. L. 1942 Spore formation by *Bacillus subtilis* in peptone solutions altered by treatment with activated charcoal. J. Bact., **44**, 653–659.
87. ROUX, E. 1890 Bactéridie charbonneuse asporogène. Ann. inst. Pasteur, **4**, 25–34. Cited by Migula.
88. SCHMIDT, C. F. 1950 Spore formation by thermophilic flat sour organisms. I. The effect of nutrient concentration and the presence of salts. J. Bact., **60**, 205–212.
89. SCHREIBER, O. 1896 Ueber die physiologischen Bedingungen der endogenen Sporenbildung bei *Bacillus anthracis, subtilis* and *tumescens*. Zentr. Bakt. Parasitenk., Abt. I., Orig., **20**, 353–374, 429–437.
90. SNEDECOR, G. W. 1946 Statistical Methods, 4th Ed., Iowa State College Press, Ames, Iowa.
91. STUMBO, C. R., MURPHY, J. R., AND COCHRAN, J. 1950 Nature of thermal death time curves for P.A. 3679 and *Clostridium botulinum*. Food Technol., **4**, 321–326.
92. SUGIYAMA, H. 1950 Studies on factors affecting the heat resistance of spores of *Clostridium botulinum*. Ph.D Thesis, University of Chicago, Chicago. Also J. Bact., 1951, **62**, 81–96.
93. TARR, H. L. A. 1933 Some observations on the respiratory catalysts present in the spores and vegetative cells of certain aerobic bacilli. Biochem. J., **27**, 136–145.
94. VINTON, C., MARTIN, S., JR., AND GROSS, C. E. 1947 Bacteriological studies relating to thermal processing of canned meats. VII. Effect of substrate upon thermal resistance of spores. Food Research, **12**, 173–183.
95. VIRTANEN, A. I. AND PULKKI, L. 1933 Biochemische untersuchungen über bakterien-sporen. Arch. fur Mikrobiologie, **4**, 99–122.
96. WEIL, R. 1901 Arch. Hyg., **36**, 205 *et seq.* Cited by Fischoeder.
97. WEISS, H. 1921 The heat resistance of spores with special reference to the spores of *B. botulinus*. J. Infectious Diseases, **28**, 70–92.
98. WILLIAMS, O. B. 1929 The heat resistance of bacterial spores. J. Infectious Diseases, **44**, 421–465.
99. WILLIAMS, O. B. AND HARPER, O. F., JR. 1951 Studies on heat resistance. IV. Sporulation of *Bacillus cereus* in some synthetic media and the heat resistance of the spores produced. J. Bact., **61**, 551–556.
100. WILLIAMS, O. B. AND REED, J. M. 1942 The significance of the incubation temperature of recovery cultures in determining spore resistance to heat. J. Infectious Diseases, **71**, 225–227.
101. WITHELL, E. R. 1942 The significance of the variation in shape of time survivor curves. J. Hyg., **42**, 124–183.
102. WUND, M. 1906 Feststellung der Kardinalpunkte der Sauerstoffkonzentration für Sporenkeimung und Sporenbildung einer Reihe in Luft ihren ganzen Entwicklung-

gang durchführender, sporenbildender Bakterienspecies. Zentr. Bakt. Parasitenk., Abt. I, Orig., **42**, 97–101, 193–202, 289–296, 385–393, 481–488, 577–588, 673–688.

103. WYNNE, E. S. 1948 Physiological studies on spore formation in *Clostridium botulinum*. J. Infectious Diseases, **83**, 243–249.
104. WYNNE, E. S. AND FOSTER, J. W. 1948 Physiological studies on spore germination with special reference to *Clostridium botulinum*. I. Development of a quantitative method. J. Bact., **55**, 61–68.
105. WYNNE, E. S. AND FOSTER, J. W. 1948 Physiological studies on spore germination with special reference to *Clostridium botulinum*. II. Quantitative aspects of the germination process. J. Bact., **55**, 69–73.
106. WYNNE, E. S. AND FOSTER, J. W. 1948 Physiological studies on spore germination with special reference to *Clostridium botulinum*. III. Carbon dioxide and germination, with a note on carbon dioxide and aerobic spores. J. Bact., **55**, 331–339.
107. WYNNE, E. S. AND HARRELL, K. 1951 Germination of spores of certain *Clostridium* species in the presence of penicillin. Antibiotics and Chemotherapy, **1**, 198–202.
108. YESAIR, J. AND CAMERON, E. J. 1936 Centrifugal fractionation of heat resistance in a spore crop. J. Bact., **31**, 2–3.
109. ZOBELL, C. E. 1946 Marine Microbiology. Chronica Botanica Co., Waltham, Mass.